MOLECULAR CHARACTERIZATION OF SEDIMENT BACTERIAL COMMUNITIES AFFECTED BY FISH FARMING

Manu Tamminen

Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Finland

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Supervisor
Professor Marko Virta
Department of Food and Environmental Sciences, University of Helsinki

Reviewers
Senior researcher Hermanni Kaartokallio
Marine Research Centre, Finnish Environment Institute

Professor Henning Sørum
Department of Food Safety and Infection Biology
Norwegian School of Veterinary Science

Opponent
Professor Søren Sørensen
Department of Biology, University of Copenhagen

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles and manuscripts, which are referred to by their Roman numerals in the text.

I Tamminen, M.; Karkman, A.; Lõhmus, A.; Muziasari, W.; Takasu, H.; Wada, S.; Suzuki, S. and Virta, M. 2011. Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. Environmental Science and Technology 45: 386-391

II Pitkänen, L.; Tamminen, M.; Hynninen, A.; Karkman, A.; Corander, J.; Kotilainen, A. and Virta, M. Fish farming affects the abundance and diversity of the mercury resistance gene merA in marine sediments. Submitted.

III Tamminen, M.; Karkman, A.; Corander, J.; Paulin, L. and Virta, M. 2011. Differences in bacterial community composition in Baltic Sea sediment in response to fish farming. Aquaculture, in press.

IV Tamminen, M. and Virta, M. 2010. Single gene-based distinction of individual microbial genomes from a mixed population of microbial cells. Submitted. Preprint available in http://precedings.nature.com/documents/4761/version/1.

AUTHOR CONTRIBUTIONS

I Manu Tamminen participated in designing and performing the practical laboratory work. He participated in interpreting the results and wrote the manuscript.

II Manu Tamminen participated in designing and performing the sampling. He carried out the statistical and the main part of the bioinformatic analyses, participated in interpreting the results and had the main responsibility in writing the manuscript .

III Manu Tamminen participated in designing and performing the practical laboratory work. He carried out the bioinformatic analyses, interpreted the results and wrote the manuscript.

IV Manu Tamminen participated in designing the experimental work. He carried out the experiments, interpreted the results and wrote the manuscript.

ABBREVIATIONS AND DEFINITIONS

16S, 23S and 30S rRNA	Ribosome components; RNA molecules of different molecular weights
BAPS	Bayesian analysis of population structure
BLAST	Basic local alignment search tool
CARD-FISH	Catalyzed reported deposition – fluorescence *in situ* hybridization
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
HELCOM	Helsinki Commission or Baltic Marine Environment Protection Commission
in situ-PCR	A polymerase chain reaction that only takes place in the vicinity of the DNA target
MDA	Multiple displacement amplification
merA	Mercury resistance gene; mercury reductase
NCBI	National Center for Biotechnology Information
nt	NCBI nucleotide database
OSPAR	Oslo and Paris Convention or Convention for the Protection of the Marine Environment of the North-East Atlantic
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
tet(A/C/E/G/M/W)	Genes for tetracycline resistance

ABSTRACT

Fish farming introduces nutrients, microbes and a wide variety of chemicals such as heavy metals, antifoulants and antibiotics to the surrounding environment. Introduction of antibiotics has been linked with the increased incidence of antibiotic resistant pathogenic bacteria in the farm vicinities. In this thesis molecular methods such as quantitative PCR and DNA sequencing were applied to analyze bacterial communities in sediments from fish farms and pristine locations. Altogether four farms and four pristine sites were sampled in the Baltic Sea. Two farm and two pristine locations were sampled over a surveillance period of four years. Furthermore, a new methodology was developed as a part of the study that permits amplifying single microbial genomes and capturing them according to any genetic traits, including antibiotic resistance genes.

The study revealed that several resistance genes for tetracycline were found at the sediment underneath the aquaculture farms. The copy number of these genes remained elevated even at a farm that had not used any antibiotics since year 2000, six years before this study started. Similarly, an increase in the amount of mercury resistance gene *merA* was observed at the aquaculture sediment. The persistence of the resistance genes in absence of any selection pressure from antibiotics or heavy metals suggests that the genes may be introduced to the sediment by the farming process. This is also supported by the diversity pattern of the *merA* gene between farm and pristine sediments. The bacterial community-level changes in response to fish farming were very complex and no single phylogenetic groups were found that would be typical to fish farm sediments. However, the community structures had some correlation with the exposure to fish farming.

Our studies suggest that the established approaches to deal with antibiotic resistance at the aquaculture, such as antibiotic cycling, are fundamentally flawed because they cannot prevent the introduction of the resistance genes and resistant bacteria to the farm area by the farming process. Further studies are required to study the entire fish farming process to identify the sources of the resistance genes and the resistant bacteria. The results also suggest that in order to prevent major microbiological changes in the surrounding aquatic environment, the farms should not be founded in shallow water where currents do not transport sedimenting matter from the farms. Finally, the technique to amplify and select microbial genomes will potentially have a considerable impact in microbial ecology and genomics.

TIIVISTELMÄ

Kalankasvatuksen yhteydessä meriympäristöön vapautuu ravinteita, mikrobeita, raskasmetalleja, pilaantumisenestoaineita sekä antibiootteja. Antibioottien sekä muiden edellä mainittujen aineiden yhteisvaikutuksesta kasvattamoiden lähiympäristössä on havaittu korkeita määriä antibiooteille vastustuskykyisiä bakteereita. Tässä väitöskirjatyössä sovellettiin molekyylimenetelmiä (kvantitatiivinen PCR ja DNA-sekvensointi) selvittämään kalankasvatuksen vaikutusta kasvattamoiden alla olevan sedimentin bakteeriyhteisöihin. Kaikkiaan neljältä kasvattamolta ja neljältä puhtaalta paikalta Itämeressä kerättiin sedimenttinäytteitä. Kahdelta kasvattamolta ja puhtaalta paikalta näytteitä kerättiin neljän peräkkäisen vuoden ajan. Lisäksi työn aikana kehitettiin uudenlainen menetelmä yksittäisten mikrobisolujen genomien monistamiseen ja seulomiseen geneettisten ominaisuuksien perusteella.

Tutkimuksessa kasvattamoiden alla olevasta sedimentistä löydettiin kohonneita määriä useita geenejä, jotka antavat vastustuskyvyn tetrasykliini-antibiootille. Kyseisten geenien määrä pysyi jatkuvasti kohonneena jopa kasvattamolla, joka oli lopettanut kaikkien antibioottien käytön jo vuonna 2000; kuusi vuotta ennen tämän tutkimuksen alkua. Samankaltainen ilmiö havaittiin myös elohopealle sietokyvynkyvyn antavalla *merA*-geenillä, jota löytyi kalankasvatukselle altistuneista sedimenteistä enemmän kuin muista sedimenteistä. Geenien korkea määrä sekä *merA*-geenin DNA-sekvenssityyppien jakautuminen viittaa siihen, että geenit saattavat tulla kasvattamoiden sedimenttiin kasvatusprosessin seurauksena. Kalankasvatus ei aiheuttanut selvästi tyypillisiä muutoksia sedimentin bakteeriyhteisöissä. Yhteisön rakenteessa tapahtui kuitenkin tietynlaisia muutoksia, jotka olivat yhteydessä viljelyn suuruusluokkaan.

Tutkimuksessa saadut tulokset viittaavat siihen, että kalankasvattamoilla perinteisesti käytetyt menetelmät, erityisesti antibioottikierto, ovat tehottomia antibiooteille vastustuskykyisten bakteerien määrän hallitsemiseksi, sillä ne eivät estä kalankasvatusprosessin aikaisemmista vaiheista tulevien resistenssigeenien ja resistenttien bakteereiden päätymistä kasvattamoille. Lisätutkimuksia tarvitaan selvittämään, mikä vaihe kalankasvatusprosessissa saa aikaan antibiooteille vastustuskyvyn antavien geenien leviämisen. Tulosten mukaan merkittävien mikrobiologisten yhteisömuutoksien välttämiseksi sedimentissä kalankasvattamoita ei tulisi perustaa matalaan veteen, missä virtaukset eivät pääse sekoittamaan sedimenttiä. Tutkimuksen yhteydessä kehitetyllä mikrobigenomien monistuksen ja seulomisen mahdollistavalla menetelmällä tulee mahdollisesti olemaan merkittävä vaikutus tuleviin mikrobiekologisiin ja –genomisiin tutkimuksiin.

1 INTRODUCTION

1.1 AQUACULTURE AND FOOD SECURITY

Aquaculture is a rapidly expanding industry that provides the world population an important protein source as the capture fisheries are getting depleted and the world population continues to grow. From the global annual production of less than one million tonnes in the 1950s, the annual production has increased to 51.7 million tonnes in 2006. In contrast the global production from capture fisheries in 2006 amounts to 89.7 tonnes and has remained relatively stagnant for the last decade (Figure 1). From the global aquaculture 54% percent consists of freshwater fish, 27% of molluscs, 9% of crustaceans, 6% of diadromous fish, 3% of marine fish and 1% of other aquatic animals (FAO, 2009).

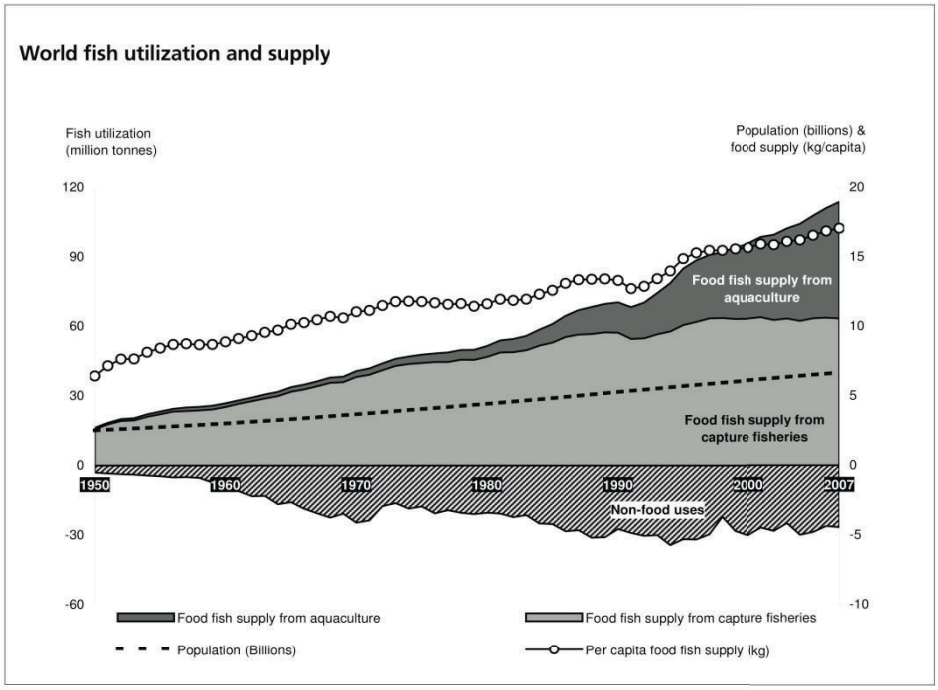


Figure 1 Food production from capture fisheries has reached a plateau around the 1990s whereas the production from aquaculture continues to grow. Modified from Figure 1 in Fishery and Aquaculture Statistics, Food and Agriculture Organization of the United Nations (FAO, 2007).

Most of the world aquaculture is performed by Asian countries. The largest aquaculture producer in world is China (34.1 million tonnes in 2006), distantly followed by India (3.1 million tonnes in 2006) and Vietnam (1.7 million tonnes in 2006) (FAO, 2009). In Europe the biggest aquaculture producer is Norway (0.7 million tonnes in 2006) with their farmed Atlantic salmon accounting for over half of the world's supply (FAO, 2009).

Aquaculture is generally believed to increase food security by compensating for the collapse of capture fisheries. Hopes also exist that by relieving the pressure on capture fisheries, aquaculture could contribute to the restoration of wild fish stocks. In their detailed analysis Naylor et al., 2000, concluded that some practices of aquaculture really do have the potential to compensate for the collapse of overfished capture fisheries, therefore contributing to an increased food security. However, due to various adverse environmental impacts, they found no support for the argument that aquaculture could contribute to the restoration of overfished capture fisheries. In the following chapters I will investigate the different environmental impacts of aquaculture practices in closer detail. In particular, I will focus on the microbiological impact of finfish farming in floating fish cages to the surrounding marine environment and the hazards to fish and human health.

1.2 ENVIRONMENTAL IMPACTS OF AQUACULTURE

The environmental effects of fish farming can be direct, as is the case with pollution emissions from the farms, or indirect, such as the contribution to overfishing by feed production. As the volume of the industry is expanding, the intensity of these effects is also expected to worsen.

The cultivated fish often escape from the fish cages causing harm to the wild fish populations (McGinnity et al., 2003). For instance, in recent years the frequency of occurrence of farmed Atlantic salmon among wild populations in the Faroese ocean area has been between 20% and 30% (Read et al., 2003). The escaped species compete with the wild species and contribute to the disappearance of local adaptive traits. Escaped fish often carry and spread parasites and diseases that are enriched at fish farms (Kent, 2000). This further contributes to the collapse of wild fish populations.

Carnivorous fish species such as salmonids are fed with fish meal- and fish oil-based feed to supply amino acids and fatty acids that the animals cannot obtain from plant protein and oil. Fish oil and fish meal are prepared from low-quality catch that is needed in substantial amounts to cultivate fish - producing one kilogram of fish requires on the average 1-3 kilograms of fish feed (Naylor et al., 2000). The requirement for fish oil and fish meal indirectly contributes to overfishing of oceanic fish stocks. Furthermore, because fish oil and meal are manufactured from fish species high in food chain, they are often concentrated in environmental toxins. Fish oil typically

contains fat-soluble agents such as PCBs, dioxins and PAHs, and fish meal is concentrated in organic forms of arsenic and mercury (Choi et al., 1998; Berntssen et al., 2010). These compounds become further enriched in the farmed fish and also accumulate to the surroundings of the aquaculture farms.

Approximately 36% of the feed is excreted as organic waste (Brune et al., 2003) and some feed will remain uneaten by the fish. This waste from the excrement and uneaten feed is rich in nutrients and still contains approximately 85% of the phosphorus, 52-95% of the nitrogen and 80-88% of the carbon that were originally present in the feed (Wu, 1995). Because most of the marine fish is farmed in floating net cages near shore that permit a free flow of excrement and unenaten feed to the surrounding water (Naylor et al., 2000; Figure 2), aquaculture causes a considerable nutrient load to the environment and contributes to the eutrophication of the surrounding marine areas. According to an estimate, a sediment area in a radius of 1 km around a farm facility is impacted by eutrophication (Wu, 1995). Severe eutrophication leads to oxygen depletion in the sea floor and subsequently to the accumulation of hydrogen sulfide and ammonia to the sediment and water (McCaig et al., 1999; Holmer et al., 1996). Oxygen depletion drastically decreases the macrofaunal species diversity of the affected sediments and waters and damages the marine ecosystems (Weston, 1990). Eutrophication has been recognized as a severe problem at least in the European Union and both OSPAR and HELCOM have highlighted the problem of nitrogen and phosphorus discharges from aquaculture operations into the North Sea and the Baltic Sea (OSPAR, 2003; HELCOM, 2004).

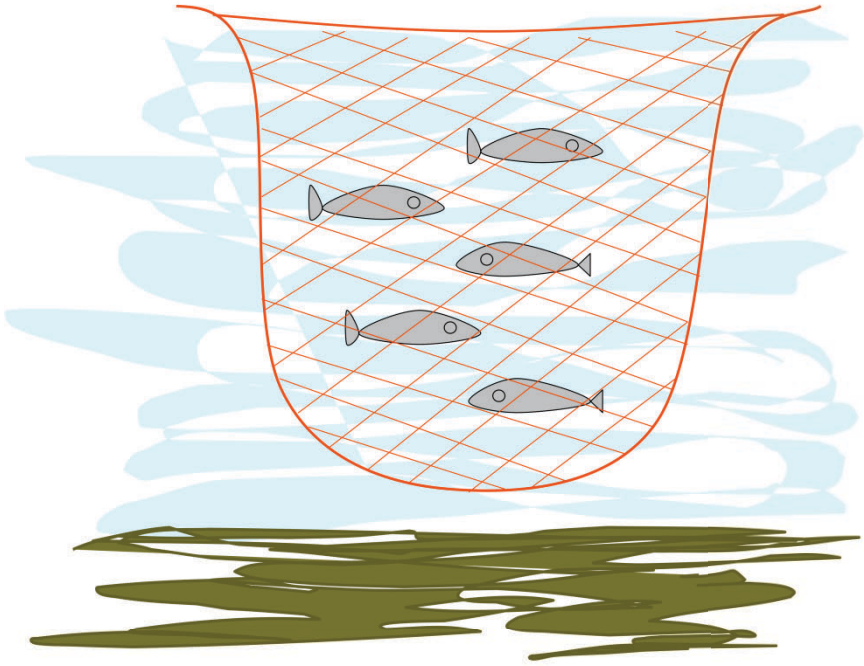


Figure 2 Fish is typically farmed in open net cages near shore where the fish are not microbiologically isolated from other cages, agricultural runoff or wastewater. The fecal matter and uneaten feed sediment under the net cages and release nutrients, chemicals and microbes to the environment. (Courtesy of Leena Pitkänen)

Aquaculture also contributes to environmental pollution by releasing substantial amounts of different chemicals directly used in culturing or contained in feed to the water, including antibiotics, antiparasitics, antifoulants and heavy metals (Burka et al., 1997; Nonaka et al., 2007; Dang et al., 2008; Agersø et al., 2007; Waddy et al., 2007; Braithwaite et al., 2007). Antibiotics and antiparasitics are needed because the fish are cultivated in high density and therefore pathogenic bacteria and parasites rapidly infect the whole fish stock if no preventive measures are taken. Several antibiotics are persistent compounds that can contaminate the environment of the aquaculture facilities as well as seafood products (Cabello, 2006). Antifoulants such as copper containing paints are used to protect the farming cages and other equipment from biofouling (Burridge et al., 2010). Finally the fish feed contains heavy metals and persistent organic pollutants that accumulate in the environment around the farms (Choi et al., 1998; Berntssen et al., 2010). Many of these compounds are harmful to macrofaunal species and some have the potential to cause undesired microbiological changes in the farm area.

Finally, aquaculture causes an enrichment of antibiotic resistant fish and human pathogenic organisms at the aquaculture locations, contributing to the global problem or antibiotic multiresistance. The following chapter will

review the different aspects of emergence of antibiotic resistant fish and human pathogenic bacteria.

1.3 MICROBIOLOGICAL IMPACT OF AQUACULTURE

1.3.1 FISH AND HUMAN PATHOGENS IN AQUACULTURE FACILITIES

Fish farming cages maintain the fish at high densities, providing conditions where contagious illnesses can spread rapidly through the farm. The farms are crowded in coastal waters and typically lack any sanitary barriers to isolate them microbiologically from other aquaculture farms, agricultural runoff or wastewater (Naylor et al., 2000). Therefore it is typical to observe an enrichment of fish pathogenic organisms in conjunction with aquaculture farms, including species such as *Vibrio anguillarum, Vibrio harveyi, Flavobacterium columnare, Aeromonas salmonicida* and *Photobacterium damselae* (Gomez-Gil et al., 2004; Pedersen et al., 2008; Pulkkinen et al., 2010). The pathogens can form a reservoir in the sediment around the farming cages, as demonstrated with fish pathogen *Vibrio salmonicida* (Enger et al., 1989; Husevåg et al., 1991).

Aquaculture farms provide conditions that can enrich also human pathogens. Some of the pathogens naturally occur in the marine environment and include species such as *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Listeria monocytogenes, Clostridium botulinum,* and *Aeromonas hydrophila* (Dalsgaard, 1998; Feldhusen, 2000; Tamplin, 2009; Vandenberghe et al., 2003; Igbinosa et al., 2008). A connection exists between the enrichment of fish pathogens and human pathogens as some fish pathogenic *Aeromonas* strains are also emergent human pathogens (Dalsgaard, 1998; Jacobs et al., 2007). Other human pathogens are introduced into the marine environment and aquaculture systems by contamination from wastewater or agriculture, including *Salmonella* spp., *Escherichia coli, Shigella* spp., *Campylobacter* spp. and *Yersinia enterocolitica* (Dalsgaard, 1998; Feldhusen, 2000). Human pathogenic organisms have often been found in seafood products and they are typical causes of gastroentritis, especially in conjunction with the consumption of uncooked seafood (Dalsgaard, 1998; Igbinosa et al., 2008; Wong et al., 2000; Butt et al., 2004; Daskalov, 2006; Bhowmick et al., 2008).

1.3.2 ANTIBIOTIC RESISTANT PATHOGENS AT AQUACULTURE FACILITIES

Antibiotics are regularly used to combat bacterial infections at the fish farms. The antibiotic compounds are typically fed to the fish by mixing them with fish feed. A large part of the antibiotics remains unmetabolized by the fish

and ends up in the surrounding environment through fecal contamination. According to estimates performed at a Greek fish farm, up to 73% of oxytetracycline fed to the fish can remain in the feces (Rigos et al., 2004). Many antibiotics are persistent compounds that accumulate in the environment of the aquaculture facilities (Cabello, 2006). As a result, aquaculture has been observed to increase the amount of antibiotic resistant bacteria in the sediment around the farm facilities (Ervik et al., 1994; Nonaka et al., 2007; Seyfried et al., 2010) and even in wild fish and mussels captured around fish farming facilities (Ervik et al., 1994). Antibiotic use has also lead to the emergence of antibiotic resistant fish pathogens, causing complications to antibiotic chemotherapy (L'Abée-Lund, 2000; Schmidt et al., 2001; L'Abée-Lund et al., 2002a; L'Abée-Lund et al., 2002b; Sørum et al., 2003; Akinbowale et al., 2006; Agersø et al., 2007; Jacobs et al., 2007).

Recently, some antibiotic resistance determinants identified in human pathogenic bacteria have been traced back to aquatic environment. A quinolone resistance determinant that has been observed in eg. *Klebsiella pneumoniae, Escherichia coli, Enterobacter cloakae* and *Enterobacter sakazakii* has been identified to originate from a nonpathogenic marine bacterium *Shewanella algae* (Poirel et al., 2005). Resistance genes for florfenicol and tetracycline in *Salmonella enterica* serotype *Typhimurium* DT104 are also likely to originate from marine bacteria (Bolton et al., 1999; Angulo et al., 2000). Similarly, a transfer of sulfonamide and tetracycline resistance genes from a fish pathogen *Aeromonas salmonicida* to *Escherichia coli* has been demonstrated (L'Abée-Lund et al., 2002a; Sørum et al., 2003). A high similarity of tetracycline resistance genes between bacterial isolates from a fish farm and clinical bacterial isolates has also been demonstrated (Furushita et al., 2003). These examples provide evidence that aquaculture farms provide an environment that promotes the transfer of resistance genes from marine organisms to human pathogens. Indeed antibiotic resistant pathogenic organisms have been found in seafood products (Wong et al., 2000; Ottaviani et al., 2001). Furthermore, even nonpathogenic resistant bacteria present in seafood products cause a risk to the consumers because their antibiotic resistance genes may become a part of the human commensal microbial flora and eventually transfer to opportunistic pathogenic organisms (Salyers et al., 2004).

1.3.3 ANTIBIOTICS IN AQUACULTURE AND THEIR RESISTANCE MECHANISMS

To understand the emergence and spreading of antibiotic resistance at aquaculture farms and seafood, several factors have to be considered. These include pollution of the aquaculture sites by antibiotics and heavy metals, the physiological and genetic properties of antibiotic resistance, the microbes

present in the fish farm microbial community, and finally the hygiene aspects related to transfer of the resistant strains to food products.

Typical classes of antibiotics used in finfish aquaculture include tetracyclines, sulphonamides, penicillins, quinolones, beta-lactams, macrolides, nitrofurans and amphenicols (Burka et al., 1997; Sapkota et al., 2008). A part of the antibiotics end up in the sediment through digested or undigested fish feed. Although the concentration of the antibiotics in the sediment varies and is not necessarily high enough to cause a selection pressure for antibiotic resistant bacterial strains, the presence of sub-therapeutic antibiotic amounts has been observed to promote horizontal transfer of resistance genes in the microbial communities (Ghosh et al. 2007). In addition to antibiotics, other chemicals such as heavy metals and biocides are known to contribute to the effect of antibiotics in promoting horizontal transfer of antibiotic resistance genes (Chapman, 2003; Baker-Austin et al., 2006). Particularly mercury has been found in elevated amounts in fish feed and has been observed to promote the selection and transfer of antibiotic resistance genes (Choi et al., 1998; Baker-Austin et al., 2006). Fish farming therefore introduces chemicals in the environment that promote horizontal transfer of resistance genes and that are potentially selective for antibiotic resistant bacterial strains.

Antibiotics affect the microbial cells by inhibiting central metabolic functions, eg. synthesis of cell wall or protein synthesis. Microbes develop resistance mechanisms to counter the effects of antibiotics. In some cases the resistance can be intrinsic due to physiological properties of a cell (Taylor-Robinson et al., 1997). More typically the resistance is due to a mutation (Martinez et al., 2000) or horizontal acquisition of genetic markers that confer resistance to one or several antibiotics (Hawkey et al., 2009). Typical classes of antibiotics, their mechanisms of action and typical resistance mechanisms are presented in Table 1.

Table 1. *Typical antibiotics used in aquaculture, their mechanisms of action and typical resistance mechanisms against them.*

Antibiotic group	Mechanism of action	Resistance mechanisms	Found in mobile elements	References
Sulfonamides	Inhibition of folate synthesis by competitive inhibition of dihydropteroate synthase	Mutation in dihydropteroate synthase. Acquisition of a mutated form of dihydropteroate synthase (or a part of it).	Yes	Sköld, 2000 L'Abée-Lund et al., 2002; Sørum et al., 2003

Tetracyclines	Inhibition of translation by inhibiting the binding of aminoacyl-tRNA to the mRNA-ribosome complex	Efflux of tetracycline, ribosomal protection, enzymatic drug inactivation	Yes	L'Abée-Lund et al., 2002; Sørum et al., 2003; Rice et al., 2005; Roberts, 2005
Penicillins	Inhibition of peptidoglycan synthesis	Enzymatic drug inactivation, mutation of protein components	Yes	Rice et al., 2005; Drawz et al., 2010
Quinolones	Inhibition of DNA gyrase and topoisomerase IV	Efflux, DNA gyrase protection, mutations in DNA gyrase and topoisomerase IV	Yes	Strahilevitz et al., 2009; Richter et al., 2010
Nitrofurans	Bacterial DNA damage	Mutations in nitro-reducing activity	No references found	Raether et al., 2003; Sandegren et al., 2008
Macrolides	Inhibition of protein synthesis by preventing peptidyltransferase from adding the peptidyl-tRNA to the next amino acid	Post-translational methylation of 23S ribosomal RNA, enzymatic drug inactivation, drug efflux	Yes	Franceschi et al., 2004; Szczepanowski et al., 2007
Aminoglycosides	Interfering with DNA proofreading in replication, inhibition of ribosomal translocation, disrupting cell membrane	Enzymatic drug inactivation, efflux, mutation of 30S ribosomal subunit, methylation of aminoglycoside binding site	Yes	Tauch et al., 2002; Shakil et al., 2008

Amphenicols	Inhibition of peptidyl transferase on the 50S ribosomal subunit	Enzymatic inactivation, efflux, target mutations	Yes	Trieu-Cuot et al., 1992; Schwarz et al., 2004; Kehrenberg et al., 2005

Intrinsic resistance and the resistance acquired by mutation can only transfer vertically, from parent to progeny. However, the horizontally acquired resistance determinants move freely even between distantly related bacterial species (Frost et al., 2005). They are of special concern for human health due to their ability to rapidly disseminate resistance genes to various members in the microbial community, including pathogenic bacteria.

1.3.4 HORIZONTAL TRANSFER OF RESISTANCE GENES

Antibiotic resistance genes are transferred in mobile genetic elements along with other resistance genes eg. for mercury, virulence genes and other metabolic genes (Toussaint et al., 2002). The three main groups of mobile genetic elements are conjugative plasmids, integrating conjugative elements and bacteriophages (Frost et al., 2005) that transfer through mechanisms of conjugation (conjugative plasmids and integrating conjugative elements), transduction (bacteriophages) and transformation (conjugative plasmids, random DNA). An overview of these elements and mechanisms is presented in Figure 3. The mobile genetic elements have a mosaic-like structure and are composed of modular units, including integrons, insertion sequences and genomic islands that are often shared between the three groups (Toussaint et al., 2002; Osborn et al., 2002).

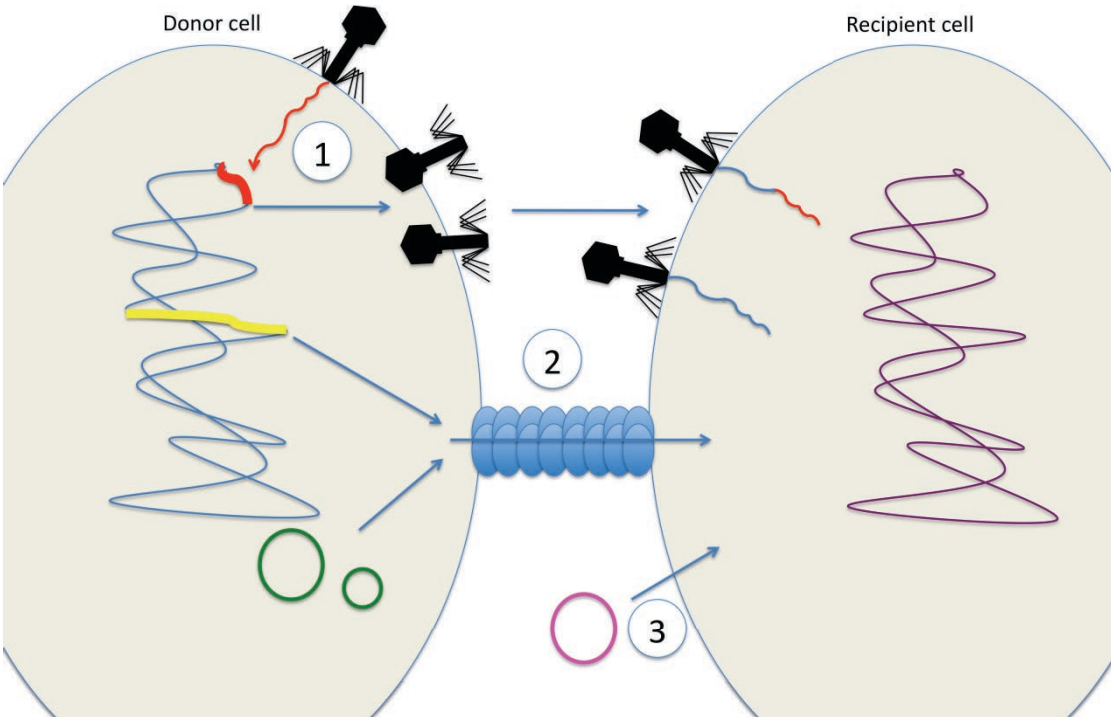


Figure 3 Transduction (1). The DNA genome (red) of a temperate phage inserts into the chromosome (blue) as a prophage. It later replicates with the chromosome, packing host DNA, lyses the cell and infects naive recipient cell in which the novel DNA recombines into the recipient host chromosome (purple). Conjugation (2). Conjugative plasmids (green) and integrated conjugative elements (yellow) use a protein structure called pilus to connect two cells and transfer DNA to recipient cell. Transformation (3). DNA in the environment (pink) is taken up by competent cells. Some of the acquired DNA may become stabilized in the new host cell. Modified from Figure 1 in Frost et al., 2005.

Conjugative plasmids are circular or linear double-stranded DNA molecules that exist extrachromosomally and replicate independently (for a review, see Frost et al., 2005). They consist of an essential backbone of genes that encode replicative functions and a variable amount of different accessory genes that can include genes for resistance, virulence or different metabolic functions. Several plasmids are known to harbor antibiotic resistance genes and transfer them between related bacterial species.

Plasmids are normally transferred by conjugation, a mechanism where a donor cell actively participates into the transport of DNA to a related microbial species. In the initial step of conjugation the two cells form a mating pair where the cells are organized in an adjacent position and a specialized pore is formed between them. In the subsequent step a signalling event occurs that initiates the DNA transfer to the recipient cell (Schröder et al., 2005). In addition to transfer of plasmids, conjugation is used in the mobilization of integrating conjugative elements (for a review, see (Burrus et al., 2004)). These chromosomally integrated elements contain genes that provide them mobility but also include genes for different functions such as

antibiotic resistance and virulence. In some cases also other chromosomal material is transferred in a conjugation event (Frost et al., 2005).

Bacterial viruses or bacteriophages are the most abundant and most rapidly replicating life forms on earth. Genomes of bacteriophages can be composed of either single-or double-stranded RNA or DNA and range by size from few to several 100 kb. Bacteriophages can be virulent, rapidly replicating and lysing the infected cells, or lysogenic, integrating to host the genome and replicating along with the chromosome until the lytic cycle is activated by some external signal (Brussow et al., 2004). Typically in lysogeny the phage genome integrates into the host genome and replicates with it as a so called prophage. This lysogenic conversion can provide the host with new phenotypic properties such as toxin production (Canchaya et al., 2003). Recombination with other prophages and other mobile elements contributes to the mosaic structure of the phage genomes (Hendrix, 2003). In the lytic cycle of the bacteriophages, host cell DNA can accidentally be packaged into the phage virion and later be transferred to a new host upon infection. This process is known as transduction (Miller, 2001). Transduction is known to relocate sections of microbial chromosomes and also be involved in dissemination of antibiotic resistance genes (Blahova et al., 1999).

In addition to conjugation and transduction, a third mechanism exists for horizontal gene transfer that permits DNA to be transferred even between distantly related bacteria. Transformation is a mechanism where extracellular DNA is taken into microbial cells that have developed a physiological state called competence (Chen et al., 2004). Competence is triggered by different environmental conditions such as cell density or starvation (Thomas et al., 2005). Several species of bacteria and archaea have been observed to be capable of this function, suggesting its importance in utilizing extracellular DNA as a source of nutrition and genetic information. The uptaken extracellular DNA has to be stabilized in the new host cell to cause genetic changes. This may occur through self-sustained replication as is the case with plasmids or through integration into the host chromosome by homologous recombination (Thomas et al., 2005).

The mosaic-like nature of the mobile genetic elements has been observed to cause accumulation of multiple resistance determinants for antibiotics and heavy metals into single mobile elements (Garriss et al., 2009). This also provides one explanation for the observed co-selection between heavy metal and antibiotic resistance (Baker-Austin et al., 2006). Marine sediment has been estimated to be an environment that promotes horizontal gene transfer (Stewart et al., 1990) and the presence of antibiotics, antifoulants and heavy metals further promotes the likelihood of the gene transfer events (Baker-Austin et al., 2006). Many of the microbial species that aqcuire the resistance genes are not likely to be human or fish pathogens. However, some bacterial strains that are harmless as such can pose a risk due to their ability to store, combine and disseminate several resistance determinants to opportunistic pathogens (Salyers et al., 2004).

The transfer of resistance genes between bacterial species depends on the bacterial species that are present in the system with the mobile genetic elements. The following chapter will review the literature on microbiological impact of fish farming and aquaculture, attempting to describe the typical bacterial species present in fish farms.

1.3.5 COMPARISON OF MICROBES BETWEEN FARM AND PRISTINE SEDIMENTS

Fish feces have rich microbiota and constantly inoculate the sediment with a diverse microbial pool, originating from the gut microflora (Ringø et al., 1998; Pond et al., 2006). The microbial community composition of the sediment below fish farms therefore results from the microbes that are native to the sediment and the microbes that are introduced from fish feces and feed. In salmonids the commonly occurring intestinal bacteria include genera *Lactococcus, Citrobacter, Shewanella, Pseudomonas, Enterobacteriaceae, Arthrobacter, Carnobacter, Aeromonas, Mycoplasma, Acinetobacter* (Ringø et al., 1998; Holben et al., 2002; Huber et al., 2004; Pond et al., 2006; Romero et al., 2006; Hovda et al., 2007; Kim et al., 2007; Ringø et al., 2008; Navarrete et al., 2009; Merrifield et al., 2009; Navarrete et al., 2010), belonging to phyla *Gammaproteobacteria, Firmicutes* and *Actinobacteria*. Some bacterial phyla, including *Fusobacteria, Firmicutes* and *Actinobacteria*, could also be introduced from the fish feed (Navarrete et al., 2009). Many of these bacteria could survive and contribute to the microflora in the sediment. In comparison, the presence of phyla *Alpha-*, *Delta-* and *Gammaproteobacteria*, *Bacteroidetes, Acidobacteria* and *Planctomycetes* have been detected in pristine antarctic marine sediments (Bowman et al., 2003a; Bowman et al., 2003b; Bissett et al., 2006; Baldi et al., 2010), *Delta-* and *Gammaproteobacteria* in arctic Atlantic sediments (Ravenschlag et al., 1999) and *Alpha-*, *Delta-* and *Gammaproteobacteria*, *Bacteroidetes* and *Actinobacteria* in arctic Pacific sediments (Li et al., 2009).

A comparison of one harbor and one shellfish aquaculture farm in Japan revealed a ubiquitous presence of the classes *Delta-* and *Gammaproteobacteria* at both sampling locations and additionally phyla *Planctomycetes* and *Crenarcheota* at the farm site (Asami et al., 2005). They furthermore observed an increase in sulfate-reducing and sulfide-oxidizing bacteria at the aquaculture farm, indicating an accelerated sulfur cycle. A comparison of three different Chinese aquaculture farms revealed a presence of *Alphaproteobacteria*, *Bacteroidetes* and *Actinobacteria* at all farm locations (Wei et al., 2009). A comparison of two fish farms and two pristine sites in Tasmania revealed a ubiquitous presence of classes *Delta-* and *Gammaproteobacteria* and *Bacteroidetes* across the pristine and farm sites. In addition to these classes, *Alpha-* and *Epsilonproteobacteria* were encountered at the farms (Bissett et al., 2006). A similar result was reported by (Kawahara et al., 2009), who also noted an increase in the amount of

Deltaproteobacteria in the sediment below the fish farms. It must be noted that the bacterial community comparison on broad phylogenetic levels (such as the levels of phylum or class presented in the examples above) is not very accurate or informative because of the diversity of ecological roles in these phylogenetic classes.

1.3.6 EUTROPHICATION AND EFFECT OF FISH FARMING ON THE NUTRIENT CYCLES

Fish farming leads to an increased amount of organic carbon, nitrogen and phosphorus in the environment around the farms (Brune et al., 2003; Piedrahita, 2003; Gutierrez-Wing et al., 2006). This has been observed to lead to local anoxia and changes in the cycles of sulfur, nitrogen and phosphorus (McCaig et al., 1999; Holmer et al., 1996; Asami et al., 2005; Ingall et al., 2005; Kawahara et al., 2008; Chavez-Crooker et al., 2010). High organic load leads to an increased amount of heterotrophic bacteria at the farm sites (Michaud et al., 2006). The breakdown of organic matter by bacteria in the sediment and bottom water consumes oxygen and eventually leads to hypoxic or anoxic conditions at the sediment around the aquaculture farms (Gray et al., 2002). Anoxia and hypoxia have considerable consequences to the aerobic life in the sediment, causing a decrease in the amount of macrofauna (Weston, 1990). The hypoxic or anoxic conditions in the sediment affect locally the biogeochemical cycles of sulfur, nitrogen and phosphorus. These changes have further significance to the biology and chemistry of the sediment.

In oxic sediments the microbes use oxygen as the terminal electron acceptor of their metabolism. When the oxygen is depleted and the sediment becomes anoxic, sulfate-reducing bacteria start using sulfate as the terminal electron acceptor (Holmer et al., 1996; Kawahara et al., 2008). This will cause an increase in the level of sulfide and hydrogen sulfide in the sediment and the bottom water. Sulfide is toxic to many life forms and therefore further alters the sediment biology by decreasing the amount of macrofaunal species (Weston, 1990; Chavez-Crooker et al., 2010).

Because the fish feed is rich in protein, its breakdown results in considerable amounts of ammonium. In pristine sediment the ammonium would be oxidized into nitrites and nitrates by nitrifying bacteria and finally reduced into nitrogen gas by denitrifying bacteria (Arrigo, 2005). However, the nitrification process is very sensitive to the low levels of oxygen and elevated levels of ammonia and hydrogen sulfide (McCaig et al., 1999; Conley et al., 2009; Crab et al., 2007). Furthermore, a high organic load causes the heterotrophic bacteria to outcompete the nitrifying bacteria (Michaud et al., 2006). This leads to accumulation of ammonium in the surrondings of fish farms. Ammonium is also toxic to many life forms, further changing the biology and chemistry of the sediment.

Anoxic conditions have the potential to solubilize phosphorus from organic matter and sediment, although the relationship between anoxia and phosphorus release is complex (Conley et al., 2009; Ingall et al., 2005). The release of phophorus could further promote the growth of heterotrophic bacteria but the overall effect of the potential phosphorus release is unclear.

The combined effect of anoxia, ammonification and sulfide accumulation could be one explanation for the decrease in bacterial species diversity in the aquaculture-impacted sediments that has been observed in some studies (Torsvik et al., 2002; Bissett et al., 2006). The implications of the decrease have not been studied to close detail but they could indicate a disruption in the fundamental biological processes of the sediment microbial ecosystem.

1.3.7 CONTAINING THE MICROBIOLOGICAL IMPACT OF FISH FARMING

The established practices to prevent the emergence of antibiotic resistant bacterial species are typically based on reducing the antibiotic use at the farms. This requires discovering alternative solutions to treating or preventing the fish diseases. One already established alternative to antibiotic treatment is using vaccinations against the illnesses (Samuelsen et al., 2006). Although it considerably reduces the need for antibiotic use, it is considered by some a relatively expensive and laborious approach and has therefore not been adapted everywhere. Another approach to reduce the need for antibiotic treatment is the use of pre- and probiotics in fish feed to enhance the immunity of the farmed fish (Gatesoupe, 2008). In a recent study Tamminen et al. (2011a) observed that tetracycline resistant microbes are found even beneath a fish farm that has not used any antibiotics for several years. This result suggests that the tetracycline resistance genes are introduced to the sediment by the fish farming process. Therefore the entire workflow of fish farming from feed and hatchling production until the cage farming has to be evaluated to reveal parts that are the sources of the antibiotic resistant bacteria. This is discussed in closer detail in the results and discussion part of this thesis.

Waste filtration techniques have been implemented to combat the eutrophicating effects of aquaculture (Piedrahita, 2003). A further technique to reduce the amount of waste to the surrounding marine environment includes integrated multi-trophic aquaculture, where several species are living in the aquaculture system and the waste produced by one species is used as nutrient by the next (Troell et al., 2009). Tamminen et al. (2011b) observed that major bacterial community-level changes can be prevented by founding the fish farms in locations where currents can move and mix the sediment beneath the farms. However, this does not prevent the contamination of the sediment beneath the aquaculture farms with antibiotic resistant bacteria.

Considerable gaps remain in our knowledge in resistance gene transfer networks between different bacterial species in complex bacterial communities. Therefore it remains problematic to design strategies to contain the resistance gene transfer and to evaluate the factors contributing to multiresistance emergence. In the following chapters I will evaluate existing and emerging methods to study complex bacterial communities and the flow of different genes between the community members.

1.4 CURRENT METHODS TO STUDY THE MICROBIOLOGICAL IMPACT OF FISH FARMING AND THEIR LIMITATIONS

1.4.1 THE CULTIVATION BIAS

Recent studies have shown that traditional microbiological culturing techniques are insufficient to address the immense microbial diversity that is present in any environmental setting (Torsvik et al., 2002). According to a famous estimate, on the average only around 1% of all microbes in the environment can be easily cultivated using the traditional cultivation approaches (Amann et al., 1995), and that the majority of environmental bacteria are classified as uncultured. It has to be clarified here that the word uncultured is typically defined to refer to a microbe that has not been cultured in a specific experiment (Handelsman, 2004). Therefore it can refer to a microbe that has not been attempted to be cultured or to a microbe that has been recalcitrant to traditional cultivation approaches but could possibly be cultivated using more advanced approaches.

The reasons for cultivation bias are diverse. The reasons include lack of necessary symbionts, nutrients, or surfaces, excess inhibitory compounds, incorrect combinations of temperature, pressure and atmospheric gas composition, accumulation of toxic waste products from their own metabolism, intrinsically slow growth rate and rapid dispersion from colonies (Handelsman, 2004; Simu et al., 2004). It is theoretically possible to take into account these considerations and design a cultivation strategy for a microbe that has previously been recalcitrant to cultivation. However, this approach would require some prior knowledge of the bacterium and its natural habitat, which is often difficult to obtain. To avoid these difficulties, approaches have been developed that focus directly on microbial DNA in the environment without attempting to solve the culturing problem.

1.4.2 DNA SEQUENCING FROM THE ENVIRONMENT

Introduction of commercial kits has made DNA extraction from natural samples such as soil, sediment or water a routine approach. The extracted DNA can be used directly for sequencing, leading to a research direction

called metagenomics (Handelsman, 2004). However, it is often desired to target the sequencing effort into certain DNA sequences in the isolated DNA, such as metabolic or 16S ribosomal RNA genes. This can be achieved by a combined PCR and sequencing, using the environmental DNA pool as a template. When designing primers for such analyses, it has to be remembered that most genes in complex microbial populations are highly diverse. Therefore it is essential to design the PCR primers to target only the conserved areas of the genes. Failure to do so will result in a biased amplification of only certain sequence types in the environmental DNA pool.

The PCR amplicons can be subjected to library construction and Sanger sequencing to reveal their diversity. More recently the 454 pyrosequencing technology with the increasing read lengths are challenging the Sanger sequencing, permitting the highly parallel sequencing of individual amplicons without a need for library construction (Margulies et al., 2005). The most commonly used application of the PCR amplification and sequencing from a complex environmental DNA pool is the phylogenetic profiling using 16S ribosomal RNA genes as markers for phylogeny of individual microbes.

1.4.3 STATISTICAL AND PHYLOGENETIC ANALYSES OF DNA SEQUENCES

The DNA sequences obtained from environmental samples contain a high amount of evolutionary and phylogenetic information. Phylogenetically particularly informative are highly conserved and slowly mutating genes whose sequences can be considered as name tags for different microbes (Santos et al., 2004) . The most used example of such genes is the 16S ribosomal RNA (16S rRNA) gene that contains a very suitable distribution of conserved and variable regions to permit primer design to target a large diversity of organisms while containing a high amount of phylogenetic information.

The phylogenetic information provided by the 16S rRNA or other housekeeping gene sequences can be used to compare bacterial community diversity and structure between different samples. To permit such comparisons, the diverse 16S rRNA genes need to be aligned to identify the corresponding nucleotides. The alignment can be done automatically by comparing the sequences to a database of previously aligned 16S rRNA sequences (DeSantis et al., 2006) . The differences between these nucleotides can subsequently be used to cluster groups of sequences according to a high shared similarity (Schloss et al., 2005; Tang et al., 2009) or to infer the phylogenetic relationships of these sequences (Guindon et al., 2003; Felsenstein, 2005). Furthermore, the sequence information can be used to determine the phylogenetic affiliation of the 16S rRNA sequences (Cole et al., 2009).

Phylogenetic clustering of the 16S rRNA genes can be used to break a large set of 16S rRNA genes into clusters with a high internal similarity. This is especially useful when studying environmental samples where the microbial diversity is high. The clustering can be based on the phylogenetic distances of the 16S rRNA genes as utilized eg. in DOTUR (Schloss et al., 2005). More elaborate Bayesian clustering methods can combine information from several conserved genes, such as BAPS (Tang et al., 2009) and generally have a higher sensitivity for the fine structure of the DNA sequence differences. Finally, different samples can be compared according to the distribution of phylogenetic clusters between them.

Multiple methods have been developed to analyze the phylogenetic information present in the DNA sequences. These range from crude but computationally light methods such as neighbor joining and maximum parsimony to advanced and sensitive but computationally more intense maximum likelihood and Bayesian methods that generally permit a deeper resolution of the phylogeny (Huelsenbeck et al., 2001; Guindon et al., 2003; Felsenstein, 2005; Stamatakis et al., 2005). Recent advances in phylogenetic inference include AdaptML, a method that uses maximum likelihood phylogeny in conjunction with information about sampling locations to infer projected habitats on the extant and ancestral nodes on the tree (Hunt et al., 2008). The projected habitats are a manifestation of ecological niches that cannot be directly observed for microbes. The identification of projected habitats adds a new layer of information on traditional maximum likelihood phylogeny as they reveal similarly adapted microbial groups between different samples.

1.4.4 SINGLE CELL APPROACHES TO MICROBIOLOGY

The cultivation-independent methods contain two serious drawbacks when compared to the traditional microbial cultivation methods. First, the molecular methods typically detect only the predominant community members (Pedrós-Alió, 2006). This seriously limits the applicability of molecular methods to investigate the rare microbial species that consitute most of the microbial diversity in any environment. Second, the genetic targets that are amplified from the isolated DNA pool lose their genetic linkage to the other genetic properties. This makes it impossible to determine eg. which functional gene and which 16S ribosomal RNA gene originate from the same microbe. These problems are widely recognized and therefore recent years have witnessed a considerable innovation in the attempts investigate single microbial cells in their natural environments. These single cell approaches permit the investigation of rare members of microbial communities (Marcy et al., 2007a). Furthermore, the single cell methods permit linking microbial functions with their phylogeny without culturing the bacteria (Stepanauskas et al. 2007). Particularly single cell approaches have shown a great promise in obtaining information from microbes in their

natural habitats, including genetically linked information from several loci. The single cell approaches include both cultivation dependent and independent approaches.

A notable approach in culture dependent single cell techniques came from Zengler et al. (Zengler et al., 2002), who trapped individual microbial cells into low-melting agarose droplets using an emulsion technique. These microscopic agarose droplets function as growth chambers for individual encapsulated cells. Because the microdroplets are impermeable to microbes, they can be suspended into different environmental matrices to provide nutrients and other beneficial conditions to the agarose-trapped cells. After incubation in the ambient temperature, the microdroplets with cell growth can be recovered using sorting flow cytometry. The method therefore permits the recovery of microscopic pure cultures for subsequent sequencing.

Culture independent single cell methods are also based on separation of the single cell into individual compartments for subsequent manipulation steps. These compartments can be microscopic chambers on a microfluidic chip (Ottesen et al., 2006; Marcy et al., 2007a; Marcy et al., 2007b), or wells on a microwell plate (Stepanauskas et al., 2007). In the microfluidic approach by Ottesen et al., 2006, the cell suspension is partitioned into microscopic chambers on a microfluidic chip. The distribution of the cells in the compartments follows Poisson distribution and therefore it is much more common to obtain a chamber with one cell than with multiple cells. These chambers are subsequently queried for a 16S ribosomal RNA gene and a target metabolic gene using a multiplex PCR reaction with two Taqman probes. The amplicons are isolated using micromanipulation from chambers where both genes has been amplified and subjected to sequencing. This approach permits linking a metabolic function to the phylogeny of 16S ribosomal RNA genes, albeit with considerable manual labor. Furthermore, the methodology does not permit recovery of multiple genetic markers from individual cells.

The approaches by Stepanauskas et al., 2007, Marcy et al. 2007a and Marcy et al., 2007b, take the concept further by performing whole genome amplification for the single cells in the compartments. They take advantage of the multiple displacement amplification that is based on random priming of a highly processive strand displacing phi29 polymerase (Dean et al., 2001). This reaction has been shown to amplify micrograms of DNA using even individual microbial genomes as templates (Stepanauskas et al., 2007; Woyke et al., 2009; Rodrigue et al., 2009). The amplified genomic DNA can be used to sequence multiple genes from a single microbe or even for complete genome sequencing. The approach by Stepanauskas et al., 2007, is based on flow cytometric sorting of individual cells on a microwell plate. The cell lysis and DNA amplification are subsequently carried out in the microplate wells. The method is in principle widely adaptable because of the abundance of cell sorters in research facilities.

The approach by Marcy et al., 2007b, uses chambers on a complex microfluidic chip to provide the reaction compartment for whole genome amplification. A great advantage of this approach is that it is much less prone to contamination because the microfluidic chambers are isolated from the external environment. Furthermore, a smaller reaction volume was observed by the authors to lead to a higher uniformity in amplification of different regions of the template DNA. The drawback of this methodology is that it requires a highly specialized microfluidic platform to be carried out.

All of the single cell methods described here suffer from the lack of high throughput selection for the genomes. The environmental microbial communities are often highly diverse which implies that the microbes containing genetic properties of interest are often relatively rare in the community. Although the single cell methods permit analysis of rare members of microbial communities (Marcy et al., 2007a), they do not currently permit targeting the sequencing effort into any particular rare subpopulation. Considering the low throughput of the microfluidic approach by Marcy et al., 2007b, and the high risk for contamination in the flow cytometric approach by Stepanauskas et al., 2007, an effective screening of the less prominent community members remains a true challenge.

2 AIMS OF THE STUDY

Aquaculture has been practiced in the Baltic Sea since the 1970s (Honkanen et al., 2000). Aquaculture in the Baltic Sea is in considerably smaller scale compared to the Asian countries and consists primarily of farming rainbow trout (*Oncorhynchus mykiss*) and common whitefish (*Coregonus lavaretus*) in net cages. From the countries around the Baltic Sea, the biggest producers of farmed fish are Finland with a production of 13 400 tonnes in 2008 (FGFRI, 2008) and Sweden with a production of 6 512 tonnes in 2003 (FAO, 2003). Altogether 85 million people live in the drainage basin of the Baltic Sea and many of the surrounding countries practice large-scale industrial and agricultural activities. Therefore the Baltic Sea is under heavy nutrient loading and in its current state highly eutrophic (HELCOM, 2009). Baltic Sea has no tide or tidal currents and therefore the impact areas of fish farms can be considered relatively isolated from other such areas. Antibiotic resistance at the farms is recognized as serious problem by the Baltic fish farmers. Apparently the antibiotic use is poorly supervised and the practices to deal with resistance consist mainly of antibiotic cycling (personal communication with the farmers). Using the fish farm sites in the Baltic Sea as target research environments, I attempted to answers the research questions presented below. The approaches are listed below and described in closer detail in the attached papers (referred to using Roman numerals I-IV).

Question 1. Does fish farming increase the number of resistance genes in the sediment beneath the farms?

Approach: Collect samples from fish farm and pristine control area sediments over four successive summers. Measure the prevalence of resistance genes using quantitative PCR. Described in I and II.

Question 2. What is the origin of the resistance genes?

Approach: Sequence the amplicons of the quantitative PCR reactions to determine the diversity of the amplicons. Analyze the sequences using phylogenetic tools to compare the phylotype distribution between fish farm and pristine sites. Described in II.

Question 3. Does fish farming cause typical bacterial community-level changes in the sediment beneath the farms?

Approach: Amplify and sequence multiple 16S ribosomal RNA genes from fish farms and pristine sites. Identify the typical 16S phylotypes at fish farms using statistical and phylogenetic tools. Described in III.

Question 4. Which bacteria harbor resistance genes at the fish farms?

Approach: Develop a single cell-based method to identify and separate the resistant community members in a cultivation-independent fashion. Described in IV.

3 SUMMARY OF METHODS

3.1 SAMPLING LOCATIONS

Sediment samples were collected during 2006-2009 from FIN1 and FIN2 farm sites, two medium-scale fish farms in the northern Baltic Sea in the Turku Archipelago. In addition, SWE1 and SWE2 farm sites corresponding to small- and medium scale farms in the Stockholm Archipelago were sampled in August 2007. The sampling locations are described in Table 2. The top 5 cm of surface sediment was collected using an Ekman grab (Envco, Romania) for FIN1 and FIN2 farm and pristine sites during 2006, and using a Sediment limnos (Limnos Ltd, Turku, Finland) for all sites during 2007-2009. The sediment samples were immediately frozen on dry ice until DNA extraction at the laboratory. The DNA was extracted using a commercial FastDNA spin kit for soil (Qbiogene, Morgan Irvine, CA, USA).

Table 2. *Sampling sites and their characteristics.*

Sampling site	Farmed fish	Production (tons year^{-1})	Other notes
FIN1 farm site	Common whitefish, rainbow trout	50	Situated in a shallow bay. Use of antibiotics stopped ~2000.
FIN1 pristine site	None	None	Situated in a shallow bay; sampled 1000 m from FIN1 farm site.
FIN2 farm site	Rainbow trout	50	Situated in a deep bay with a strong current. Use of antibiotics stopped ~2000; florfenicol used occasionally.
FIN2 pristine site	None	None	Situated next to a large open water area with a current. No fish farms are located within a radius of several kilometers.
SWE1 farm site	Rainbow trout	5	Situated in a shallow bay. Florfenicol used occasionally.
SWE2 farm site	Rainbow trout	50	Situated in a shallow bay. Florfenicol used occasionally.

3.2 METHODS

The techniques and methods used in this study are listed in Table 3. A more detailed description of each method is provided in the original manuscripts.

Table 3. *The methods that were used in the study. The Roman numerals refer to the original papers in which the methods were applied.*

Method	Manuscript
Sampling	I, II, III
DNA extraction from the sediment	I, II, III
Determination of physicochemical parameters from the sediment samples	I, II, III
Determination of tetracycline and oxytetracycline residues from the sediment samples	I
Determination of heavy metal concentrations of the sediment samples	II
Primer design for tetracycline resistance genes	I
Primer design for mercury resistance gene	II
Quantitative PCR measurements	I, II
Library sequencing of tetracycline resistance genes	
Library sequencing of mercury resistance gene	II
Phylogenetic analyses of the resistance genes	II
Library sequencing of 16S ribosomal RNA genes	III
Phylogenetic and statistical analyses of 16S ribosomal RNA genes	III
BLAST analyses of 16S ribosomal RNA genes	III
Microbial extraction from sediment	IV
Microbial capture to polyacrylamide droplets	IV
Construction of picoreactors for DNA amplification	IV
DNA amplification in picoreactors	IV
Labelling of picoreactors according to a target gene	IV
Flow cytometric identification of the labelled picoreactors	IV

4 RESULTS AND DISCUSSION

4.1 QUANTITATIVE ANALYSES OF RESISTANCE GENES

We tested the persistence of several resistance genes at the aquaculture farms by using PCR and quantitative PCR on the sediment samples collected at the Baltic fish farms over the course of four years. Furthermore, we tested the distance to which the resistance genes spread from the farms. For this PCR and qPCR was used to measure the presence and copy number of resistance genes in pristine control sediments sampled near the fish farms. First, new PCR primers were designed for tetracycline resistance genes *tetA*, *tetC*, *tetE*, *tetG*, *tetM* and *tetW* and and for the β/γ-*Proteobacterial* family of mercury resistance gene *merA*. The primer sequences can be found in I for the tetracycline resistance genes and II for the *merA* gene. The primers were designed based on the most comprehensive and most up-to-date sequence data available at the time to account for the sequence diversity of the resistance genes. All the designed primers amplified the desired products without significant side products or primer dimers. The amplicons were confirmed to be correct by sequencing and BLAST analysis using blastn algorithm with default settings against NCBI nt database.

Sediments under FIN1 farm site contained the tested tetracycline-resistance genes at most sampling times (Figure 4A). Most of the genes were not detected outside the farm; even the nearest sample (200 m from the farm) showed no signals for the tetracycline resistance genes. Most of the genes tested were also found at the FIN2 and SWE2 farm sites, whereas sediment under SWE1 farm site contained only *tetC* and *tetM* (Figure 4B). The samples collected outside these farms contained no *tet* genes above detection limit. *merA* genes were present at all sampling sites at least on some time point but their amount was constantly elevated at the fish farm sediments over the course of four years. Bacterial numbers were relatively similar between the sampling locations and times as indicated by the copy number of 16S rRNA genes.

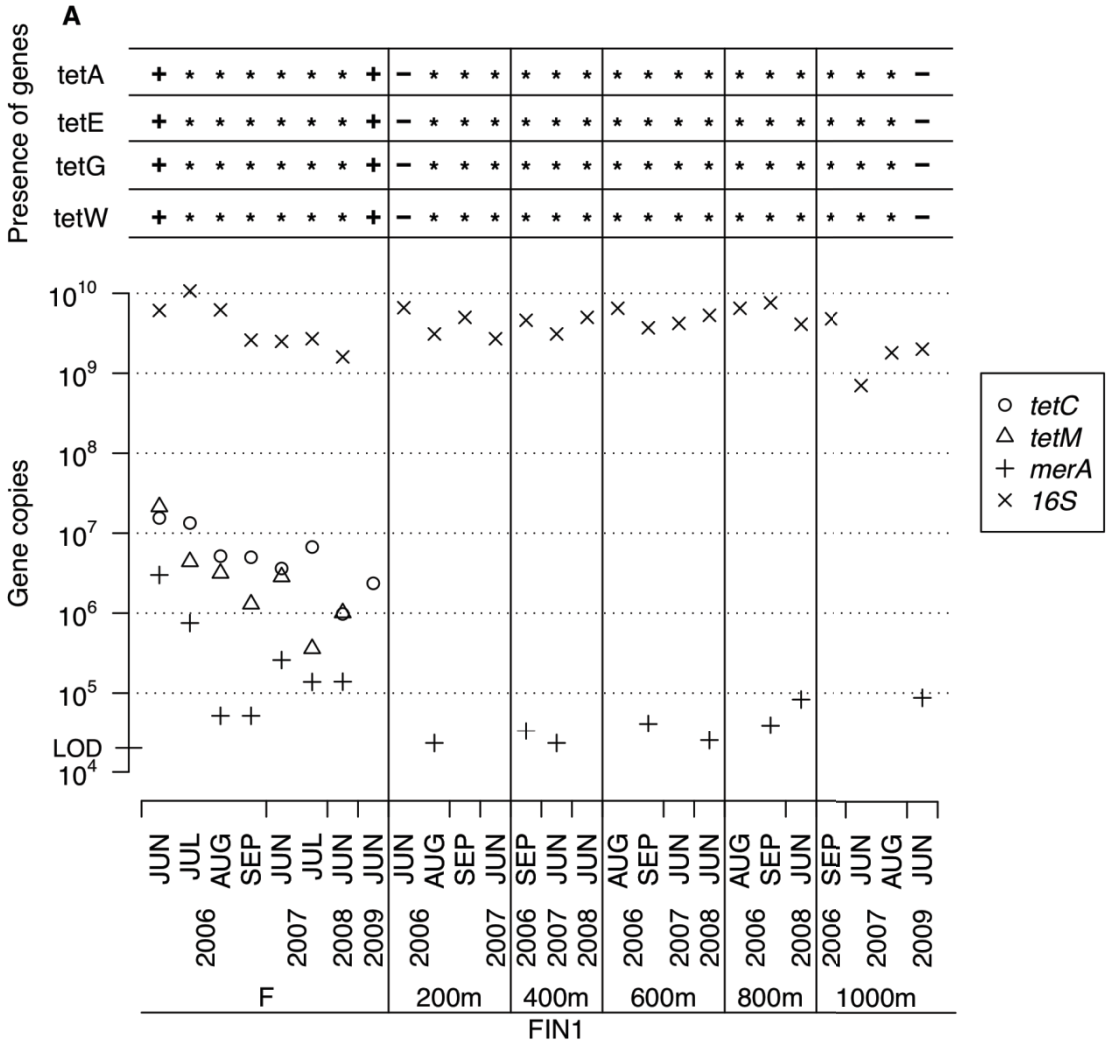
Because there was no evidence for a decrease in copy number of the resistance genes during the 4-year observation period at any of the sampling locations, the selection pressure for the resistance genes was measured. Tetracycline and oxytetracycline were measured using bioreporter assay and HPLC analysis in all sediments (for details, see I). Mercury content of the sediment was measured using a bioreported and a pyrolytic method. The tetracycline and mercury concentration of the samples was too low to cause any kind of selection pressure (for details, see I and II). Presence of tetracycline, oxytetracycline, mercury or other heavy metals cannot therefore

explain the high persistent copy numbers of the resistance genes at the farm sites.

At the Baltic fish farms, the persistence of the resistance genes likely results from one or more of the following reasons. The resistance genes could be subject to cross-selection eg. by other antibiotics (Dang et al., 2007) or heavy metals (Baker-Austin et al., 2006). This is because several resistance genes are often present in mobile elements, as described in 1.3.3. As specific selection pressure leads to selection of the whole mobile element, the selection therefore includes all the other resistance genes present in the element. The resistance genes could also be introduced by a constant influx of resistant bacteria from external sources, such as farmlands and wastewater treatment plants (Dang et al., 2009), fish hatchlings (Rhodes et al., 2000) or fish feed (Seyfried et al., 2010; Kerry et al., 1995). However, the origin or the resistance genes is probably not from agriculture or communal waste because the archipelago surrounding the farms is sparsely inhabited and unused for agriculture. A more likely source is fish feces. Although the fish gut is not a particularly well-studied environment, the animal gut is generally considered a hotspot for horizontal transfer and enrichment of resistance genes (Shoemaker et al., 1992). Coselection with antibiotics and mercury, often present in low amounts in fish feed (Choi et al., 1998), and increased horizontal gene transfer rate typical to animal gut, results in an environment where different resistance genes are likely to spread to several different types of bacteria.

Although the number of the resistance genes remained elevated in the sediments under fish farms, the prevalence of these genes decreased rapidly outside farms; even samples collected 200 m from farms contained no detectable levels of any of the tetracycline resistance genes tested (Figures 4A and B). The lack of diffusion of the genes is unexpected, considering the continuous presence of the genes at the farms and the free flow of seawater that could spread the gene-harboring bacteria and lead to colonization of the surrounding sediments. Because our results in the Baltic Sea suggest that the resistance genes do not easily spread to the surrounding environment from the fish farms, they are therefore probably not a serious environmental problem. This is contrary to previous studies where tetracycline-resistant bacteria have been cultivated from Mekong river sediments in Vietnam (Kobayashi et al., 2007; Suzuki et al., 2008) and marine sediments in Japan, including sediments from pristine sites (Rahman et al., 2008) and sediment sampled close to an aquaculture farm (Nonaka et al., 2007). The difference between these studies and the present study could be due different laboratory techniques used (the differences between cultivation-based and molecular techniques have been discussed in chapters 1.4.1, 1.4.2 and 1.4.4). However, it also has to be considered that the potential exposure of sediments to agricultural or aquacultural runoff or wastewater is much more likely in Mekong River and Asian coastal waters than in the sparsely inhabited archipelago area in the Baltic Sea. The presence of *merA* genes at

pristine sediments has not been reported before. However, previous studies have reported the presence of *merA* genes in heavy metal-contaminated sediments (Ramond et al., 2008) and in different soil depths (Oregaard et al., 2007).



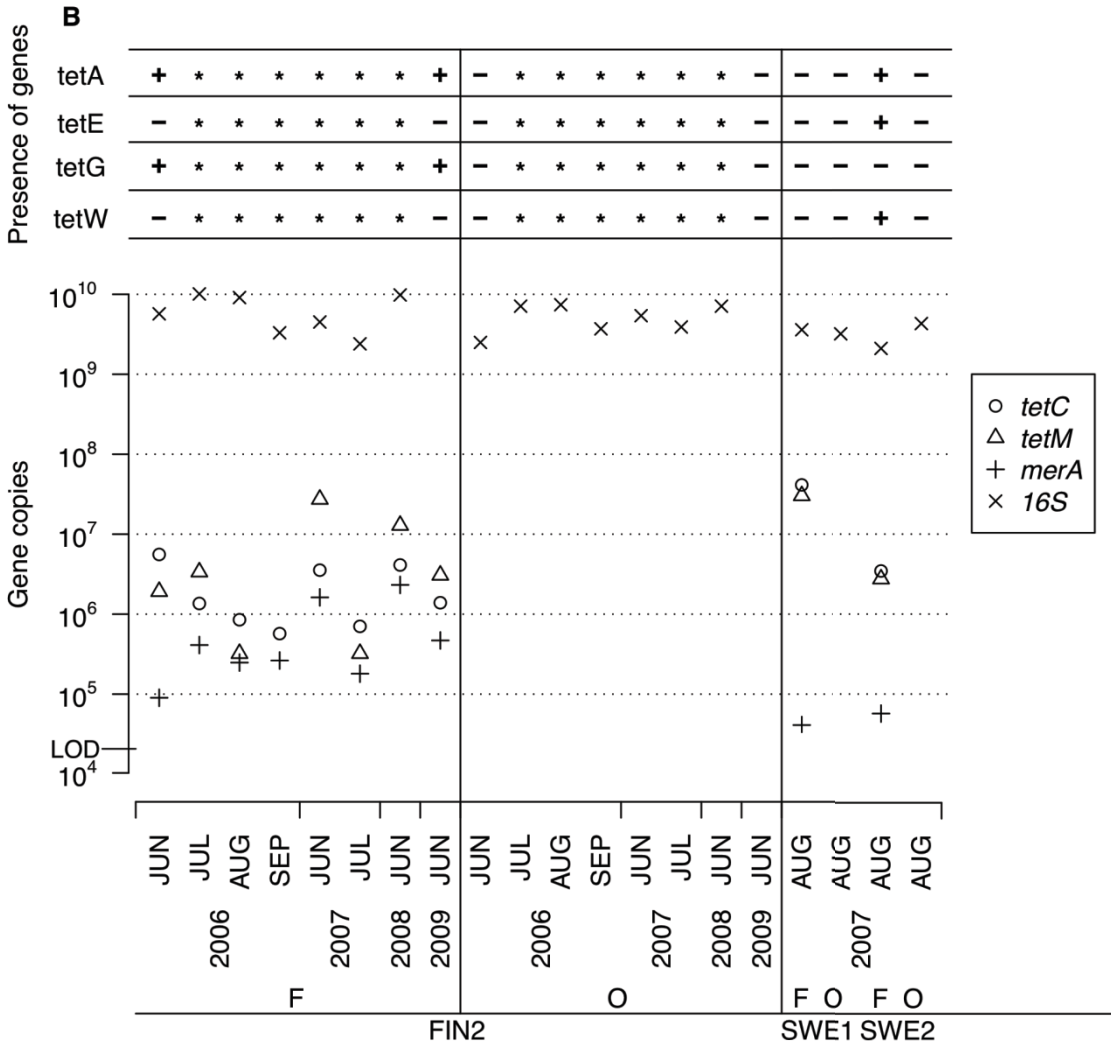


Figure 4 Presence of genes *tetA*, *tetE*, *tetG* and *tetW*, and copy numbers of *tetC, tetM*, *merA* and 16S rRNA at farms FIN1 (A) and FIN2, SWE1 and SWE2 (B). Farms FIN1 and FIN2 were sampled eight times during four consecutive summers; farms SWE1 and SWE2 were sampled once. Sediments collected at FIN1 at 200-m intervals extending from the farm boundary correspond to the observations, FIN1 200m – 1000m. FIN2, SWE1 and SWE2 correspond to the sediments collected 200 m from the respective farms. "+" denotes the presence of a gene; "-" indicates that levels were below the limit of detection; and "*" indicates that a sample was not measured.

4.2 DIVERSITY OF THE RESISTANCE GENES

The sequence diversity of the resistance genes can potentially give information about their origin. If we consider that the resistance genes are introduced from an external source, fish farms farms receiving material from the same source should have a similar diversity distribution of the gene sequences. To observe the diversity of different resistance genes at the farms, individual clones of fragments of *tetC* (unpublished), *tetM* (unpublished) and

merA (published as a part of II) were sequenced. Altogether 91 *tetC*, 310 *tetM* and 361 *merA* fragments were sequenced from farm sites.

The sequences of *tetC* from every farm site were identical apart from few occasional single nucleotide polymorphisms. It is possible that this is due to a PCR bias because all the available *tetC* sequences in NCBI were very similar. Therefore the source material for primer design may not represent accurately the sequence diversity of *tetC* genes in the environment. However, it is also possible that the *tetC* diversity in the environment really is as low as observed. The sequences available in NCBI for *tetM* and *merA* had considerably higher sequence diversity. In fact, the primer design for *merA* had to be limited to the *merA* sequences of gram-negative bacteria because of lack of sequence conservation. Consistently, the PCR amplicons of *tetM* and *merA* had higher sequence diversities than the *tetC* amplicons.

Each fish farm had a significantly unique distribution of *tetM* sequences (Figure 5, p-value < 0.001 according to X^2 tests). The results between the Finnish and Swedish farms (FIN1 and FIN2 versus SWE1 and SWE2) and between the Swedish farms (SWE1 and SWE2) are in agreement with the hypothesis about the resistance introduction by the farming process because these farms receive their feed and hatchlings from separate sources. The result is more difficult for farms FIN1 and FIN2 that receive their fish hatchlings and feed from the same source. The difference in the cultivated fish species (rainbow trout and common whitefish in FIN1 versus only rainbow trout in FIN2) potentially affects and explains the diversity pattern of the resistance genes. The different histories of antibiotic use between the farms FIN1 and FIN2, as described in Table 2, could also affect the diversity pattern. Finally, the pristine sediment could harbor a low-abundance *tetM* pool that affects the *tetM* gene diversity at the farm sites. However, this speculation will neither support nor refute the original hypothesis about the resistance gene introduction by the farming process.

The phylogenetic tree of *merA* sequences supports the division between the sequence types that are prominent at the fish farms and at the pristine sites (Figure 6). The sequence diversity between the different fish farms and between the farm and pristine sites was significantly different (p-value < 0.001 according to a X^2 test). However, the difference between the pristine sites was not significant (p = 0.16). The *merA* sequences at the fish farms consisted of sequences that had very similar BLAST matches to the nt database of NCBI (84-100%). The phylotypes at pristine sites no close matches in the nt database (57-65%; Figure 4; Supplementary information). The pristine locations therefore contain *merA* sequence types that are very different from the sequences in farm sites. This suggests that the *merA* genes at the fish farms have been introduced from an external origin.

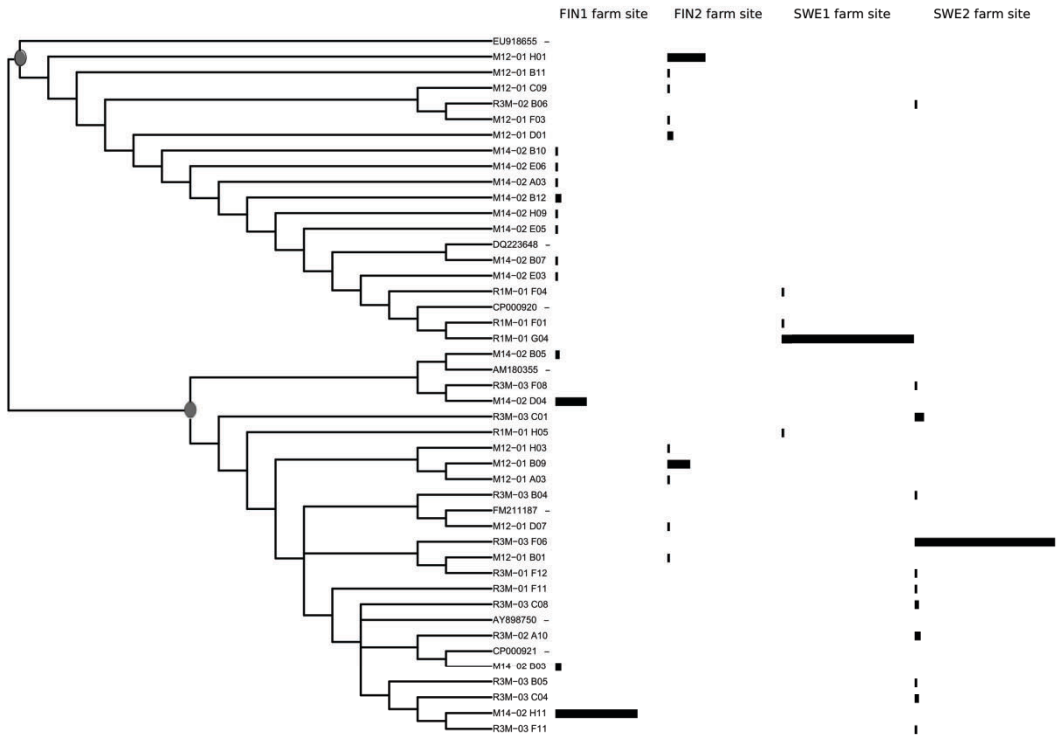


Figure 5 Neighbor joining tree of *tetM* sequences based on 1000 bootstraps. The neighbor joining tree is a cladogram in which the branches with greater than 95% bootstrap support (from 1000 bootstraps) are indicated by grey dots. Sequences are divided into two separate clades according to the major branches of the maximum likelihood tree.

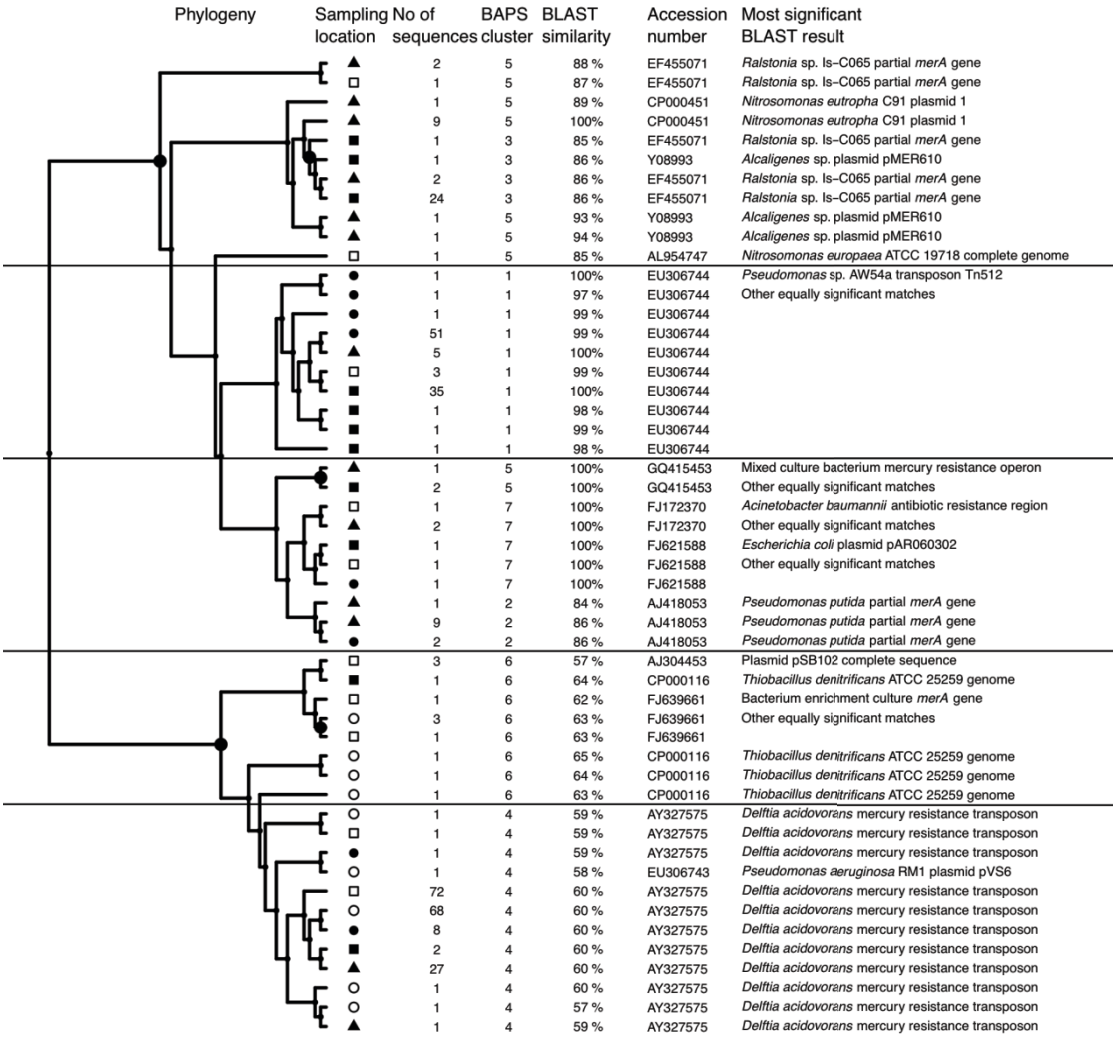


Figure 6 Maximum likelihood tree, BAPS clusters and BLAST matches of *merA* sequences. The maximum likelihood tree is a cladogram in which the branches with greater than 95% bootstrap support (from 1000 bootstraps) are indicated by black dots. Sequences are divided into five separate clades according to the major branches of the maximum likelihood tree. The sampling locations are indicated as follows: ■ FIN1 farm site, ▲ FIN2 farm site, ● SWE1 farm site, □ FIN1 pristine site and ○ SWE1 pristine site. Figure and legend are presented as a part of **II**.

4.3 BACTERIAL COMMUNITY LEVEL CHANGES IN RESPONSE TO FISH FARMING

To observe the bacterial community-level changes in response to fish farming, altogether 1153 partial 16S rRNA sequences were obtained from fish farms FIN1, FIN2, SWE1, SWE2 and pristine sites FIN1 and FIN2. The coverage of the sequencing effort was tested using richness estimates provided by DOTUR and was observed to be nearly exhaustive on the artificial phylum level of 80% sequence identity (III).

To determine the prominent bacterial phyla at fish farm and pristine locations, we assigned sequences to phyla according to an 80% confidence threshold using a naïve Bayesian classifier provided by the Ribosomal Database Project (Wang et al., 2007). Although most of the sequences were phylogenetically affiliated, a large number of sequences remained unassigned. To visualize the phylogeny of the bacterial 16S rRNA sequences, we performed a maximum-likelihood analysis using PhyML software (Guindon et al., 2003) on the 1153 sequences from fish farm and pristine sites and the sequence of *Methanococcus maripaludis* 16S rRNA (accession number BX950229), which was included as an outgroup. AdaptML (Hunt et al., 2008) was used on the maximum-likelihood tree to estimate habitats shared by different strains (Figure 7).

AdaptML estimates most likely habitats for the observed bacteria in different sampling locations. Four such habitats were predicted based on the maximum likelihood phylogeny, corresponding approximately to the FIN1 farm site (Habitat 1), the SWE2 farm site (Habitat 2), the FIN2 farm and FIN1 and FIN2 pristine sites (Habitat 3), and the FIN2 and SWE1 farm sites (Habitat 4). Medium-scale farms in shallow water (FIN1 and SWE2) correspond to two habitats that are separate from the other farms (FIN2 and SWE1) and pristine sites. On the other hand, a small-scale farm and a medium-scale farm in deep water mix together with habitats in pristine sediments. Medium-scale fish farming in shallow water therefore has an impact on the composition of sediment bacterial communities.

A clustering-based analysis was performed to detect more specific differences in sediment bacterial communities between fish farm and pristine sites. The phylum-level clustering obtained using the naïve Bayesian rRNA classifier (Fig. 3) was further refined using non-hierarchical clustering provided by a BAPS package (Tang et al., 2009). BAPS identified a partition with 20 clusters (data not shown) with a posterior probability close to 1 over multiple estimation runs. Therefore, the BAPS clusters are statistically well supported. Combining the clustering from naïve Bayesian classification and BAPS resulted in 37 phylogenetic clusters (Figure 8). To test whether the fish farms differed from the pristine sites with respect to phylogenetic cluster composition, we performed an unrooted hierarchical clustering (Figure 8). The large fish farms FIN1 and SWE2 in shallow water were separated from the pristine sites and fish farms FIN2 and SWE1 with high bootstrap values (0.97 for approximately unbiased and 0.70 for bootstrap probability values). The fish farms FIN2 and SWE1, a medium-scale farm in open water and a small farm, respectively, were not clearly distinguished from pristine sites based on their phylogenetic clusters.

Clusters that are prominent at the fish farms FIN1 and SWE2 include primarily different bacteria from the genera *Trichococcus*, *Paenibacillus*, *Acetobacterium*, *Streptococcus, Syntrophomonas* and *Clostridium* (belonging to phylum *Firmicutes*). Fish farms FIN2 and SWE1 contain increased proportions of bacteria from genus *Corynebacteria* (belonging to

phylum *Actinobacteria*) and phylum *Chloroflexi*. The strongest presence of *Gammaproteobacteria* is at the farms FIN2 and SWE1 and pristine site FIN2 whereas the large farms FIN1 and SWE2 in shallow water contain only low amounts of them. The different *Deltaproteobacteria*, *Epsilonproteobacteria* and *Bacteroidetes* clusters have been scattered across pristine and farm sites without a clear pattern. *Alphaproteobacteria* are only detected at the pristine sites. A closer analysis of the content of these clusters is provided in Table 2 of III.

Our findings suggest that medium-scale fish farming in shallow water leads to considerable changes in sediment bacterial communities. We assume that these changes result partly from introduction of bacteria by fecal matter and feed, and partly from eutrophication. The contribution of these impacts was discussed in closer detail in chapters 1.3.4. and 1.3.5. Based on the 16S rRNA sequences, we can determine the species distribution of different sampling locations and compare them using various statistical, phylogenetic and mathematical tools. However, by using these approaches, we cannot make any conclusions about the genes that these bacteria contain and therefore their specific functions in the microbial community.

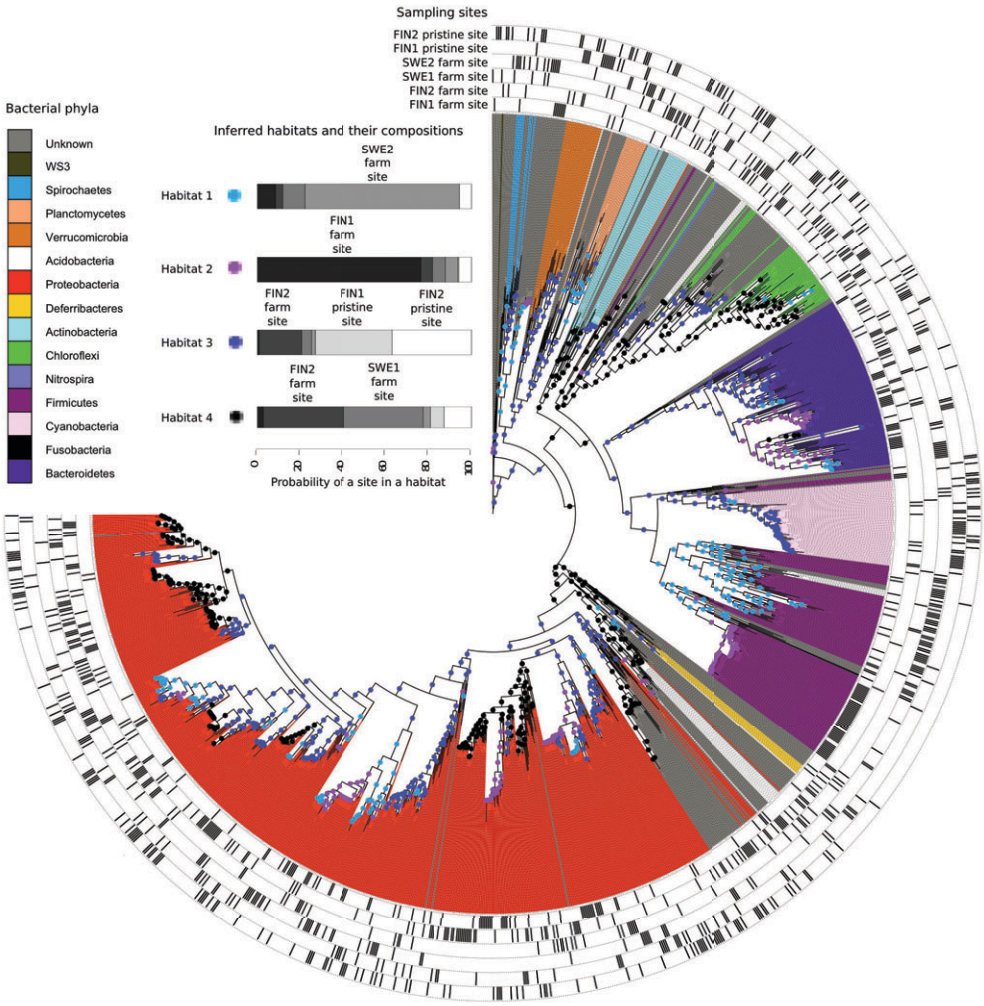


Figure 7 Sampling locations, phylum classification and habitat predictions mapped onto a maximum-likelihood tree based on 16S rRNA gene sequences from fish farm and pristine sites. Projected habitats are identified by colored dots at the nodes and leaves. The probability of encountering a strain adapted to a given habitat at different sampling locations is indicated in the colored bar-plot. Phylum classifications are indicated by colored bars extending from the tips. The presence of 16S rRNA clones in sampling locations is indicated by black lines outside the tree. Figure and legend are presented as a part of **III**.

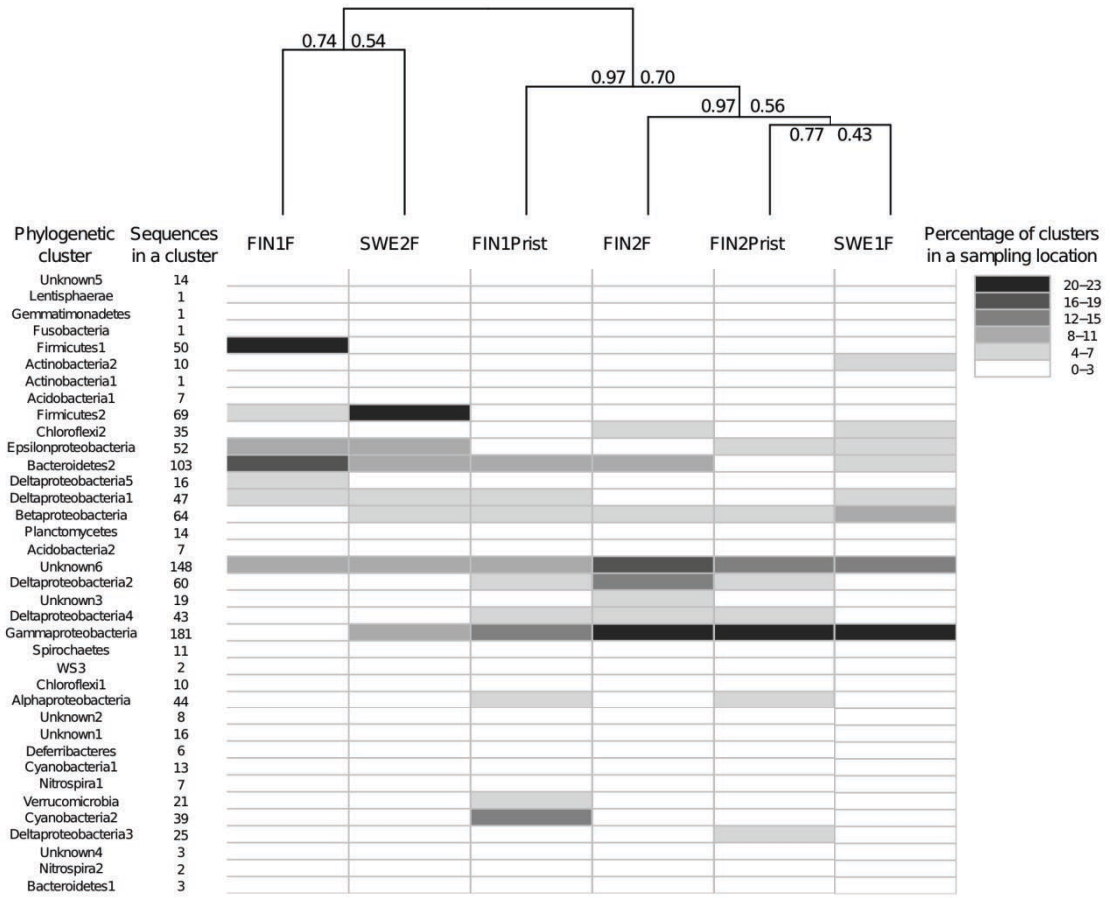


Figure 8 The presence of different phylogenetic clusters at fish farm and pristine sites. Hierarchical clustering dendrogram (based on 10000 bootstraps) is used to indicate the similarities between the sampling sites. Bootstrap support for the dendrogram according to approximately unbiased (left) and bootstrap probability (right) analysis is indicated at the nodes. The large fish farms in shallow water (FIN1 and SWE2) differ from the other farms and pristine sites with high bootstrap values (0.97 approximately unbiased and 0.70 bootstrap probability values). Figure and legend are presented as a part of **III**.

4.4 A SINGLE CELL APPROACH TO DETERMINE THE HOST SPECIES OF RESISTANCE GENES

The identities and functions of bacteria carrying specific genes in complex microbial ecosystems cannot be determined by sequencing of 16S rRNA genes or specific functional genes. Sequencing genomic DNA from individual cells in the target microbial community would permit linking specific functions to an accurate phylogeny of these microbes. However, individual genomes contain very little DNA and are therefore difficult to use for subsequent amplification or sequencing applications due to contamination from irrelevant DNA. Furthermore, selecting the genomes of interest for further analysis while excluding the noise from contamination and uninteresting community members remains problematic as the target population is often very small compared to the diversity of the complete population. To overcome these difficulties, we attempted to develop a method that combines the strengths of existing single cell techniques while excluding their weaknesses. The method consists of trapping individual microbial cells to polyacrylamide matrix, lysing the cellular structures while supporting the genomic DNA, constructing picoliter-volume reactors for genome amplification, labelling the reactors according a target gene, and finally flow cytometric selection of the labelled picoliter reactors. The schematic steps of the method can be found in IV.

To access the genetic material of a microbial cell, its cell wall needs to be permeabilized to permit the entry of enzymes and small molecules such as PCR primers, without dispersing the genome. This requirement was achieved by trapping the cells into microscopic polyacrylamide droplets that provide a support for the genomic material. To create the polyacrylamide droplets the bacterial cells were suspended into acrylamide solution and mixed into emulsion oil. In this study, a mixture of *Escherichia coli* XL1 and MC1061 cells and a marine sediment microbial community spiked with *E. coli* XL1 were used. The emulsion droplets were polymerized and the emulsion oil washed away. This resulted in individual microbial genomes trapped into discreet polyacrylamide droplets that are porous enough to permit the enzymatic destruction of the cell wall but rigid enough to hold the genome in place (Figure 9). The polyacrylamide polymer was created using a crosslinker that has sulfur-sulfur bonds and can be broken with reducing agents such as dithiothreitol.

To create amplification reactors for the genomic material, another emulsion step was used to add a layer of agarose on the droplets. The polyacrylamide was dissolved away by dithiothreitol and the droplets became

hollow agarose shells containing the genomes that had been trapped in the polyacrylamide matrix. Agarose is porous enough to permit the diffusion of multiple displacement amplification (MDA) reagents while retaining the amplified DNA. The hollow agarose reactors were suspended in an emulsion and used for highly parallel amplification of the trapped genomes (Figure 10).

In a subsequent step the picoreactors containing the genomic material with a target gene were labelled using a fluorescent dye. To achieve the labelling, a new polyacrylamide layer was added on the agarose reactors. This time the polyacrylamide matrix was prepared with an immobilized PCR primer complementary to the target gene. The target gene in this case was tetracycline resistance gene *tetB* present as a stable single copy in *E. coli* XL1 genome (Keasling et al., 1991). These droplets were subjected to a PCR reaction; an amplicon forms only on the droplets containing genomic material with the target gene of interest. Because of the covalent linkage between the primer and the polyacrylamide matrix, the resulting amplicon also remains covalently attached to the polyacrylamide matrix. The amplicons were subsequently used as targets for a fluorescent probe. Finally, the labelled droplets could be differentiated from the nonlabelled by flow cytometry (Figure 11). Populations down to 0.01% of the total cell population could be identified in the mixture of the two *E. coli* strains. In the spiked marine microbial community the autofluorescence of some particles was limiting the sensitivity of the selection but nevertheless a spiked *E. coli* XL1 population of 1% was clearly visible.

These experiments demonstrate a successful amplification, labeling and flow cytometric differentiation of individual microbial genomes based on the presence of a single-copy gene - in this case a tetracycline resistance gene present in the *E. coli* XL1 genome. The polyacrylamide that was polymerized on the microbial cells in an emulsion provides a support matrix for the genomic material. This is necessary because some cells are highly resistant to cell lysis, whereas others lyse completely, even after a brief enzymatic treatment. The technique presented in this study allows extended incubation times with high concentrations of lytic enzymes because it supports genomic DNA as a discreet package, even after the cell wall and other structures have been completely degraded. Therefore, this method can be used to lyse all cell types, from fragile to highly resistant, in the same reaction. Although the model organism in this study was a Gram-negative bacterium, we expect that the method is equally applicable to different bacterial, archaeal and eukaryotic cells. Traditionally, the different lysis reaction requirements for different cell types and the risk of complete cell lysis and genome dispersion have posed major difficulties for methods such as CARD-FISH and *in situ*-PCR, which also rely on exposing genomic DNA to enzymes (Hoshino et al., 2001; Pernthaler et al., 2002).

The agarose picoreactors provide means to perform a highly parallel and miniaturized MDA reaction. The genome-wide MDA reaction takes place

simultaneously in millions of agarose picoreactors and, assuming an optimal performance of the reaction, yields picogram amounts of clonal genomic DNA. Although not demonstrated in this study, this amount would be enough for robust reamplification to microgram quantities by a subsequent MDA reaction (Sato et al., 2004; Pan et al., 2008) or even direct pyrosequencing (Meyer et al., 2008; White et al., 2009). Although a miniaturization of the MDA reaction to the nanoliter scale has been described (Marcy et al., 2007), this approach is not widely accessible to researchers because it requires a dedicated microfluidic platform. The agarose picoreactors described in this study are easy to assemble and therefore available to any laboratory.

Through emulsion PCR and fluorescent probe hybridization, the method also offers a high-throughput procedure to screen for picoreactors with genomic DNA containing a target gene of interest. The labelled fluorescent picoreactors are differentiated from empty picoreactors or those lacking the gene of interest by flow cytometry. The screening procedure is sensitive enough to detect and capture microbial genomes in which the target gene is present in as little 0.01% of the total initial cell population. The method performs well on complex sediment microbial population spiked with low amounts of *E. coli* XL1, despite the rate of false positive events that increases with dilution (Figure 8 in IV). Until now, such target-gene screening of genomes has been done by testing single amplified genomes on 96-well plates by PCR (Stepanauskas et al. 2007), an approach that would require screening an average of 104 plates to find one target genome with a target-gene frequency of 0.01%. Clearly, a high-throughput screening procedure such as that described here would be invaluable for studying microbial communities and especially those members that are not predominant in the community.

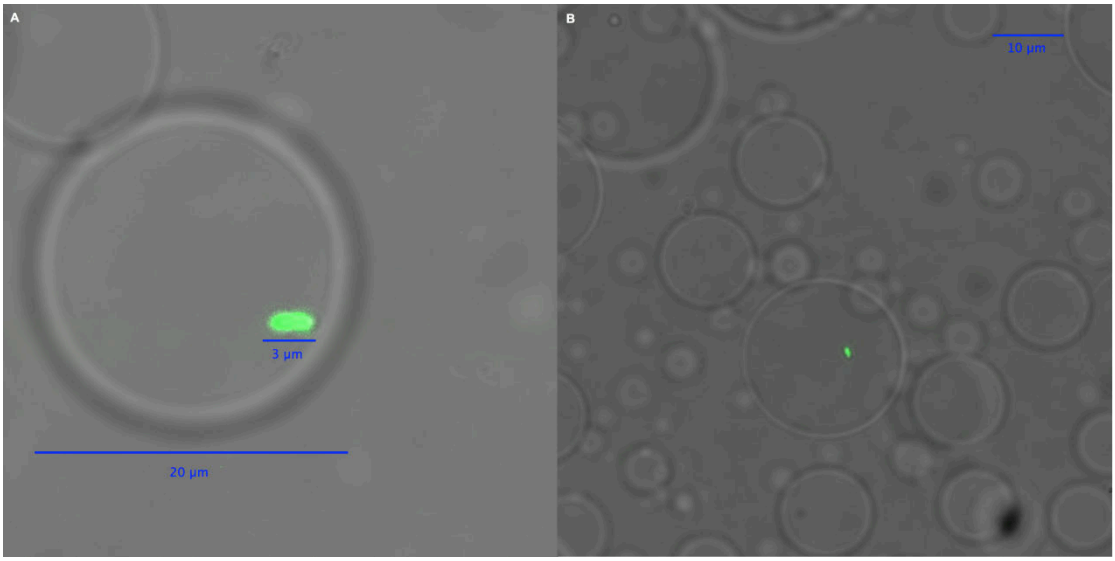


Figure 9 A differential interference contrast/confocal micrograph of (**a**) a polyacrylamide droplet containing an *E. coli* XL1 genome after cell lysis, and (**b**) several polyacrylamide droplets, one of which contains an *E. coli* XL1 genome. The green fluorescence of SYBR Green dye is used to visualize DNA. Figure and legend are presented as a part of **IV**.

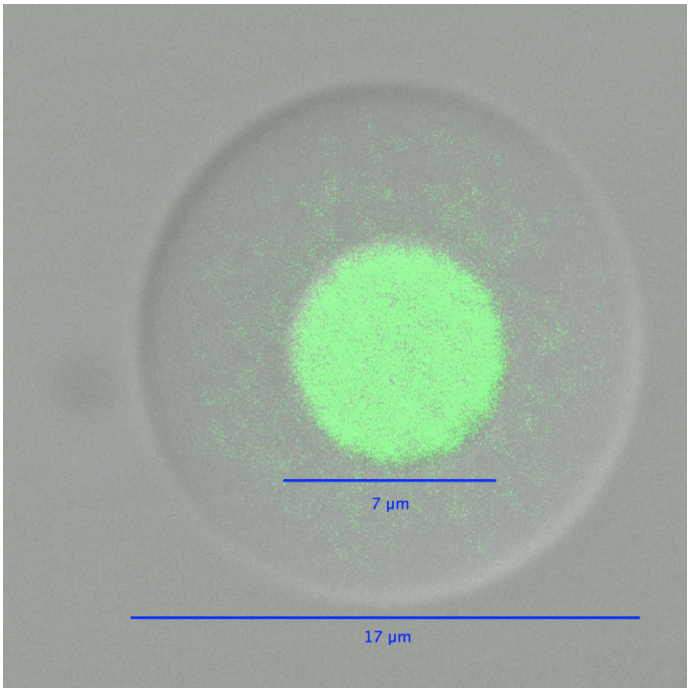


Figure 10 A differential interference contrast/confocal micrograph of an agarose picoreactor after genome amplification in an emulsion MDA reaction. The green fluorescence of SYBR Green dye is used to visualize DNA. Figure and legend are presented as a part of **IV**.

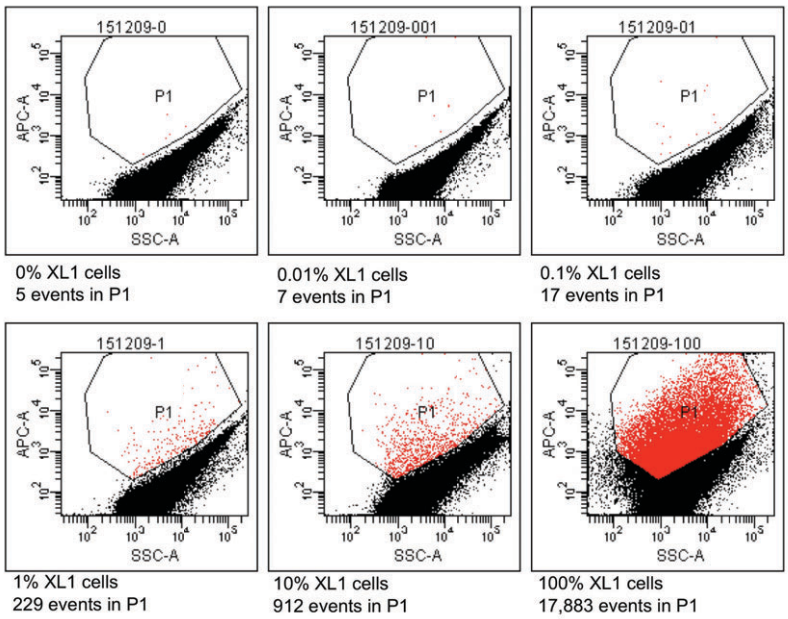


Figure 11 Flow cytometric results from picoreactors of different suspensions of *E. coli* XL1 and *E. coli* MC1061. The *E. coli* XL1 genome contains a single copy of a tetracycline resistance gene, whereas the *E. coli* MC1061 genome contains none. The genomes are amplified in agarose picoreactors and labeled by emulsion PCR and fluorescent probe hybridization targeting the tetracycline resistance gene. Picoreactors containing the XL1 genome exhibit increased red fluorescence. The parameter SSC-A on the x-axis refers to a side-scatter value that correlates with the light-scattering property of the analyzed particles. The parameter APC-A on the y-axes refers to the intensity of red fluorescence. Events in the P1 gate are labeled picoreactors containing XL1 genomes and therefore have increased red fluorescence. Altogether, 100,000 events were collected from each suspension. The fluorescent events in a suspension containing no XL1 cells (0%) are false-positive events. The non-fluorescent events in suspensions containing 100% XL1 cells represent empty picoreactors. Figure and legend are presented as a part of **IV**.

5 CONCLUSIONS AND FUTURE PROSPECTS

Ineffectiveness of antibiotic treatment against fish illnesses has been reported by Baltic fish farmers since last decade. Tetracyclines have been unusable for a decade, and a drop in the effectiveness of florfenicol is currently taking place. The typical approach to deal with antibiotic resistance at fish farms has been antibiotic cycling, where the use of one antibiotic is discontinued after resistance emergence and replaced with another type of antibiotic. After an arbitrary time the use of original antibiotic is resumed, as it is believed that the resistant bacteria against the original antibiotic have disappeared. However, our results demonstrate that resistance to tetracycline is a persistent problem at Baltic fish farms. According to our results, even several years of absence of tetracycline use will not reduce the amount of resistance genes at the sediment below the farms. Therefore the practice of antibiotic cycling appears fundamentally flawed.

To understand the reason why the resistance gene amounts remain elevated at the fish farms, tetracycline and oxytetracycline residues along with several heavy metals were quantified in the sediments. However, no significant amounts of antibiotics or heavy metals were found at the farm sediments. Therefore a likely explanation of the elevated resistance gene amounts is the introduction of the resistance genes from a source outside the aquaculture farms. The results from the diversity analysis of the mercury resistance gene *merA* give support to this hypothesis. The *merA* sequences from the fish farms resemble closely sequences from clinical isolates whereas the sequences from pristine sites have a low sequence similarity to previously known *merA* genes. The origin of the *merA* genes at the fish farms is therefore not the pristine sediment and could be explained by introduction of the *merA* genes by the fish farming process.

Assuming that the fish farming process introduces resistance genes to the farm sediment, it can also be expected that new bacterial species are introduced by the process. Sequencing of 16S ribosomal RNA genes gave insight into the differences of community structure between the farm and pristine site sediments. Although no community structure changes were detected that were similar across all the farm sediments, the overall community structure was distinct for large fish farms in shallow water. On the other hand, the bacterial community structures for a small farm and a large farm in deep water were similar to those of the pristine sites. The community changes result from a combined effect of eutrophication and introduction of microbes from fish feed and feces. Therefore the changes are very complex and bacterial species that are typical to fish farm sediments are difficult to detect.

Knowing which bacterial species contain the resistance genes would permit a deeper understanding of the origin of the resistance genes. However, current methodologies do not permit a cultivation independent analysis of bacteria harboring specific functional genes. Therefore we are unable to determine which bacteria at the fish farm sediments actually harbored the antibiotic resistance genes. The single cell method outlined in this study has the potential to answer this question in the future.

If the source of the resistance genes at the fish farms is somewhere outside the farms, it should be possible to prevent the entry of the resistance genes to the farms. Although it requires further investigation to determine the source of the resistance genes, the likely routes include fish feed and fish hatchlings. Fish feed is often enriched in environmental toxins such as mercury that can also select for antibiotic resistant bacterial species (Choi et al., 1998; Baker-Austin et al., 2006). Furthermore, the feed is often handled with the same equipment used for antibiotic containing feed. Fish hatchlings on the other hand come from cultivation facilities where they are often administered antibiotics to treat their illnesses. Therefore the fish arriving to the farm facilities could already be enriched in antibiotic resistant bacteria. Determining the sources of the antibiotic resistance genes to the fish farms will have a considerable impact on the management strategies used in finfish aquaculture.

The results presented in this study have the potential to contribute to the fish farm management strategies. The widely used antibiotic cycling strategy to combat the antibiotic resistance appears flawed in the context of our results. If the resistance genes are introduced to the farm locations from the fish feed or hatchlings, future research will be needed to pinpoint the exact source of the resistant bacteria. The single cell method developed in this study to observe the phylogenetic affiliation of antibiotic resistant bacteria could have a wider impact in microbial ecological research. It does not require any specialized instrumentation and is therefore available to any microbial ecological laboratory. It can potentially be applied to any cell types in any biological samples and can be used to target genomes according to any genes.

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